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ON GLUTOSE AND ITS BIOCHEMICAL BEHAVIOR.

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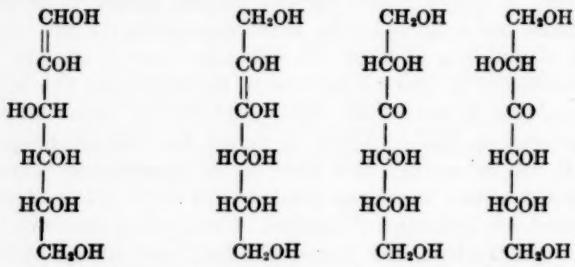
The object of the following communication is to record the results of experiments bearing upon the chemical behavior of glutose both *in vitro* and *in vivo*, including in the latter normal, diabetic, and phlorhizinized animals. Our attention was first drawn to glutose through the discovery of its formation by the action of approximately neutral sodium phosphate solution on glucose and fructose. The condition of this reaction suggested the desirability of more detailed knowledge of the biochemical behavior of glutose, which seems to have been the subject of almost no investigations, either chemical or biological, since its discovery in 1897 by Lobry de Bruyn and Van Ekenstein (1).

It will be recalled that these latter investigators showed that when a hexose sugar was treated with dilute alkali, it was converted into a mixture of isomers. Thus each of the three hexoses, glucose, fructose, and mannose is converted into an equilibrated mixture containing a certain proportion of the other two sugars, together with glutose, a non-fermentable γ-ketose. Glutose differed from the other hexoses just mentioned in that it did not yield glucose, fructose, or mannose on treatment with dilute alkali, but if acted upon at all was converted into acids, the nature of which was not specified.[1] The action of dilute alkali on glucose,

[1] L. J. Henderson (*J. Biol. Chem.*, 1911, x, 3) showed that glucose and other sugars when warmed with phosphate solutions gradually lost their optical activity, slowly at 38°, but rapidly and completely on boiling. The possibility of this reaction being due to a reversible reaction between several optically active sugars, or to a true racemization, was considered. At Professor Henderson's suggestion we have been investigating the reactions in greater detail, and are summarizing the results thus far obtained. Our experiments were mostly made by warming at 80–90° either in contact with air, or *in vacuo*, a mixture of glucose 40 gm., NaH_2PO_4 10 gm., Na_2HPO_4.

fructose, and mannose is therefore to a large extent reversible, while with glutose it is irreversible.

A brief reference must be made to the probable mode of formation of glutose, as a consideration of this question is of great significance in trying to understand the biochemical behavior of the sugar. Following a suggestion of Fischer (2) as to the possible occurrence in alkaline solution of unsaturated enolic forms of hexoses, Wohl (3) advanced the view that the equilibrium between glucose, fructose, and mannose was conditioned by a common 1.2 enolic form of the type $X \cdot C(OH) = CHOH$. Nef (4) elaborated Wohl's ideas and made a good case for the view that while a 1.2 enolic form was common to glucose, fructose, and mannose, a 2.3 enolic form derivable from fructose was the precursor of glutose which would be expected to occur in two forms designated as α and β, both of which would furnish the same osazone. The changes may be represented as follows:



1·2 enolic form common to glucose, fructose, and mannose.

2·3 enolic form yielding glutose only.

α- and β-glutose.

$12H_2O$ 45 gm., in a liter of water. In the first place it may be said quite definitely that no true racemization takes place since, on removal of phosphate and subsequent fermentation, no *l*-glucose remains behind. Secondly, in the absence of air a balanced mixture is formed containing *d*-glucose, a fair amount of mannose (isolated as phenylhydrazone), very little or no fructose (occasionally isolated as methylphenylosazone), and about 20 or 25 per cent of non-fermentable sugars chiefly made up of glutose and a little pentose. A little acid is formed even when the experiments are carried out anaerobically. When no precautions are taken to exclude access of air, much acid is produced, the main product being gluconic acid. But little lactic acid is formed under either set of conditions. At present we do not propose to investigate the reaction further.

Glutose has not yet been obtained in crystalline form and the probability that it represented more than a single individual form was realized by Lobry de Bruyn and Van Ekenstein. It differs from the ordinary hexoses mainly in its failure to undergo partial conversion into any of the fermentable hexoses under the influence of dilute alkali. Its reducing power towards Fehling's solution is only approximately half that of glucose. The occurrence of glutose in molasses was recognized by Lobry de Bruyn and Van Ekenstein, and a sample of Louisiana molasses was found to contain as much as 4.8 per cent. Pellet (5) has also investigated the question of glutose in molasses which undoubtedly originates from the action of lime on reducing sugars taking place in the refining process. Although molasses has been extensively used as feeding material for stock, the behavior of the glutose contained in it seems not to have been investigated.

The results recorded in the experimental portion of this communication show that the behavior of glutose in the animal body is in marked contrast to that of the ordinary fermentable hexoses. Absorption from the intestine is much less rapid and with large doses diarrhea is easily induced. After absorption it is found that it is utilized much less readily than the other sugars. The respiratory quotient is not altered. On giving glutose to a normal man of average size it is found that unchanged glutose can be detected in the urine even after as small a dose as 5 gm. Smaller quantities than 5 gm. seem to disappear completely, while with larger amounts usually about a quarter of the glutose is found in the urine and as much more is lost by the bowel when 40 gm. or more are consumed at one time.

On feeding glutose to diabetic patients under closely controlled conditions, it was found that no significant change occurred in the output of urinary glucose, but that a considerable amount of unchanged glutose was excreted, usually amounting to about a quarter of the quantity consumed. There was no evidence obtained in any experiment of a conversion in the diabetic organism of glutose to glucose. Experiments on phlorhizinized dogs also failed to yield any evidence of the conversion of glutose to glucose, the glucose-nitrogen ratio remaining unchanged, while the glutose administered subcutaneously was quantitatively excreted in the urine.

On administering glutose to patients on a high fat, low carbohydrate diet, the existing excretion of acetone bodies was unaffected in three subjects, though some lowering was obtained in a fourth. Furthermore, glutose was found to be of little or no value in relieving the symptoms of insulin shock in rabbits.

All of the above results tend to show how markedly glutose differs in its behavior in the living organism from the ordinary hexoses and this is the more remarkable since glutose is so readily formed from the fermentable sugars, and only differs from fructose in having its carbonyl group in position (3) instead of (2). In view of the preceding facts, it seemed worth while to make some additional experiments with glutose in order to establish further points of analogy and difference between it and related sugars.

On treating glutose with approximately normal sodium hydroxide, it was found that like glucose and fructose, it yielded large amounts of inactive lactic acid, together with a complex mixture of polyhydroxy acids. The results were much like those obtained by Meisenheimer (6) with glucose. Furthermore, comparative experiments showed that the rate of acid formation from glutose and glucose under the influence of alkali of the same concentration, did not differ much in the two cases, and certainly not enough to warrant any deductions as to the cause of the low tolerance of the body for glutose. Clearly, *in vitro*, glutose yields lactic acid as readily as glucose.

On warming glutose solutions with sodium carbonate and phenylhydrazine, it was possible to secure a good yield of methylglyoxal-bis-phenylhydrazone, so that *in vitro*, at least, there is no difficulty in resolving the six carbon chain of the glutose into three carbon substances of the type of methylglyoxal. Furthermore, on digesting glutose with zinc ammonium hydroxide, according to the method of Windaus and Knoop (7) as good a yield of methyliminazole was obtained as is furnished by any of the other hexoses. This reaction, of course, also involves the formation of methylglyoxal. In all of the foregoing changes *in vitro*, glutose simulates the fermentable hexoses as closely as possible. We have, however, observed one difference between glutose and the other hexoses which we believe may well account for its different behavior in the body. As already stated, glutose is not fermentable by yeast, and we have now found that neither living nor dead yeast has the

power to form a hexose phosphate from glutose and phosphates. In this respect glutose differs sharply from the fermentable hexoses. The work of Harden and Young (8) with yeast, and of Embden (9) and his coworkers with muscle, have substantially proved that the formation of a hexose phosphate is an essential step in the metabolic transformation of sugars both by yeast and by muscle. It would appear quite possible that the reason for the failure of glutose to ferment with yeast or to undergo rapid metabolism in muscle is to be sought in its inability to form a hexose phosphate.

Conversely, the fact that glutose, while resembling the fermentable hexoses so closely in almost all its chemical reactions save that of its ability to form a phosphoric ester, should prove to be both non-fermentable and utilized by the body only slowly or not at all, would appear to offer indirect evidence of the profound significance of hexose phosphate production in sugar metabolism. It should be noted, however, that glutose is fermentable by the colon bacillus forming both acid and gas, a change the mechanism of which is undoubtedly very different from that taking place in yeast or muscle.

EXPERIMENTAL PART.

Preparation of Glutose.

Lobry de Bruyn and Van Ekenstein describe the formation of glutose by the action of various alkalies on fructose or invert sugar. For its preparation they recommend the heating lead hydroxide (one part) with a 20 per cent aqueous solution of the hexose (two parts) for 1 hour at 100°. Lead salts are precipitated with alcohol and the residual lead removed with alcoholic tartaric acid. After removal of alcohol, the solution is fermented with pure yeast to remove fermentable hexoses, and after removal of the yeast by filtration an approximately pure glutose remains behind. We have made use of this method with success, but the employment of lead introduces an objectionable feature when the product is to be used for animal experiments, since the complete removal of the heavy metal is a difficult task. We have, therefore, sought to prepare glutose with the aid of less toxic alkalies, using sodium hydroxide, sodium carbonate, magnesia, and lime under varying conditions. The following method furnishes a product possibly

slightly less pure than that obtained by the lead method, but sufficiently good for the purposes in view. It contains no heavy metals and only small amounts of calcium salts of hydroxy acids as detectable impurities.

Cane sugar (2 kilos) is dissolved in water (8 liters) and inverted by boiling with 40 cc. of 1:10 sulfuric acid for 2 hours under a reflux condenser. Almost all (95 per cent) of the sulfuric acid is removed by adding barium hydroxide in amount previously determined by a separate experiment. Quicklime (30 gm.) of good quality is slaked in hot water and added with slaking to the invert sugar solution contained in a 12 liter flask. A cork with glass tube connection is placed in the neck of the flask which is then evacuated as far as possible. The flask is then closed while the solution is boiling vigorously under reduced pressure. In this way the disturbing influence of oxygen is largely avoided. The mixture is then immersed in a water bath and heated at 75–80° for 3 to 4 hours, with occasional shaking. After standing overnight the filtered solution will be found to be neutral and to possess only slight optical activity, usually −0.5 or less in a 1 dm. tube. 300 gm. of starch-free yeast of high purity[2] are then added and fermentation carried out at 23–25° until the evolution of gas practically ceases. The mixture is then filtered through a coarse Berkefeld filter to remove yeast and concentrated under diminished pressure. After removal of as much water as possible, the glutose is extracted with hot acetone and the solution filtered from inorganic salts, and again concentrated. The residual syrup is dissolved in water, boiled with charcoal, filtered, and concentrated to a thin syrup. The concentration of glutose in the syrup is determined by the method described in the next section. The yield averages 30 to 38 per cent of the sugar taken.[3]

[2] We are much indebted to the Fleischmann Company for liberal supplies of pure yeast.

[3] Like almost all products obtained through fermentation with yeast, it was found that the glutose preparations always gave small amounts of furfural on distillation with hydrochloric acid. The amount obtained in carefully controlled quantitative experiments corresponded to an apparent content of about 4 per cent of pentose in the glutose. Since all hexoses yield some furfural, this figure is undoubtedly too high, but whether most of the furfural comes from the glutose or from furfural-yielding impurities cannot be decided until strictly pure glutose is available for control experiments.

Quantitative Determination of Glutose.

The experiments on the fate of glutose in the body made it desirable to acquire a little more definite information about its estimation than was hitherto available. Lobry de Bruyn and Van Ekenstein state that the reducing power of glutose towards Fehling's solution is approximately half that of glucose, but it was found that a good deal depends upon the nature of the copper solution, and the time of heating. For example, using Benedict's reagent and calculating glutose as equal to twice the glucose equivalent, it will be found that the results are liable to be considerably too high. If the period of heating is short, an overestimation of 15 to 25 per cent may be made. After a number of experiments with the purest glutose that we could prepare, we found that the most satisfactory method was to proceed as follows: The glutose solution, preferably containing from 100 to 200 mg. of the sugar, is heated in a covered conical flask immersed in a boiling water bath with a good excess of the original Fehling's solution for from 15 to 20 minutes. The cuprous oxide is then filtered off on a Gooch crucible and either determined gravimetrically or by titration according to Bertrand's method. The amount of copper so obtained is converted into glucose by the use of Pflüger's table, and this multiplied by two gives a close approximation to the glutose present. Bertrand's tables for glucose were found to be less accurate for the present purpose than the older gravimetric tables of Pflüger.

For the estimation of glutose in urines containing glucose, use was made both of the optical inactivity and non-fermentability of glutose by yeast. A polarimetric estimation of glucose and a determination of the total reducing power of the urine gives a basis for a calculation of the glutose, while an independent determination was made by removing the glucose by fermentation and estimating the residual sugar by the Fehling method above described. The two methods give fairly concordant results. Seliwanoff's test was used for the qualitative identification of glutose in urine.

Fate of Glutose in the Normal Man.

These experiments were made by consuming the glutose usually in a single dose in the form of 50 per cent syrup, either immediately

after, or within an hour of breakfast, comprising no unusual excess of other carbohydrates. The results may be summarized in the following table:

Subject.	Glutose fed.	Glutose excreted by urine.	Glutose excreted by urine.	Comments.
	gm.	*gm.*	*per cent*	
Male, 74 kilos.	2.5	None.		
	3.5	Trace.		
	5	1.45	29	
	5	1.20	24	
	10	2.80	28	
	30	7.81	24	
	40	3.34	21	Slight diarrhea.
Male, 67 kilos.	50	11.12	22	Three diarrheal movements.
Female, 60 kilos.	100			Violent nausea, vomiting, and diarrhea.

In the experiments with more than 30 gm. a considerable amount of glutose was undoubtedly lost by the bowel. Absorption of glutose appears to be noticeably slow, and in the case of the experiments with 30 and 40 gm. the excretion of glutose continued for 8 and 14 hours respectively after all the glutose had been consumed. The highest percentage of glutose observed in any of the urines was 2.96, which was reached 2½ hours after a 40 gm. dose.

Additional evidence as to the slowness of absorption of glutose from the intestine was furnished in the case of a woman whose small intestine just above the cecum opened out to the abdominal wall. On giving 100 gm. of glucose by mouth in five doses 2 hours apart, but 5 gm. were recovered from the intestinal discharges; while on giving 50 gm. of glutose in four doses 2 hours apart, 20 gm. of glutose were recovered. A diabetic child was fed 54 gm. of glutose in three doses and put out 21 gm. in the stool and 15.3 in the urine. The fact that glutose was not recovered quantitatively may in part be accounted for by the fact that *Bacillus coli* formed acid and gas on 1 per cent glutose-meat infusion broth.

Fate of Glutose in the Diabetic.

Several diabetics were fed varying amounts of glutose on a diet on which they had been excreting fairly constant amounts of

TABLE I.

M. M. ♂ Age 54 years; weight 50 kilos. Diabetes mellitus.
Diet: 100 gm. carbohydrate, 60 gm. protein, and 120 gm. fat. For 4 days preceding, trace of reduction in urine. Then 50 gm. of glutose a day in 10 gm. doses.

Date.	Urine.	
	Glutose.	Glucose.
July, 1925	*gm.*	*gm.*
21–22	20.16	Trace.
22–23	14.4	"
23–24	18.52	"
24–25	0	0
25–26	0	Trace.
26–27	0	"

TABLE II.

L. K. ♀ Age 7 years; weight 19 kilos. Diabetes mellitus.
Diet: 60 gm. carbohydrate, 60 gm. protein, and 80 gm. fat.

Date.	Urine.		Glutose.	
	Glucose.	Glutose.	Fed.	In stool.
Apr., 1925	*gm.*	*gm.*	*gm.*	*gm.*
17–18	13.6	0	0	
18–19	14.6	0	0	
19–20	12.6	0	0	
20–21	11.4	15.2	58*	
21–22	13.8	0	0	
22–23	6.4	11.0	58*	
23–24	9.4	9.2	28†	
24–25	12.9	15.3	54*	21

* Three doses.
† Two doses.

glucose. A 70 per cent glutose syrup was used. The glucose excretion was practically unaffected, while considerable amounts of glutose appeared in the urine.

Fate of Glutose in the Phlorhizinized Dog.

Two dogs were phlorhizinized and given glutose subcutaneously. The results were essentially similar; in both cases the methods used showed more glutose recovered in the urine than had been injected. There was no evidence of conversion into glucose.

Effect of Glutose on the Respiratory Quotient.

Two female subjects were given glutose before breakfast. The first subject, E. M. B., weight 60.2 kilos, was normal. At 9.30 a.m. the R. Q. was 0.70. At 9.40, 100 gm. of glutose were taken by mouth. At 10.20 the R. Q. was 0.70; and at 11.05 it was 0.73.

TABLE III.

Phlorhizinized dog, ♂. Weight 7 kilos.

1.0 gm. of phlorhizin in olive oil subcutaneously daily, starting Sept. 22. No food since Sept. 20.

Date.	Urine.			
	Nitrogen.	Glucose.	Glucose : nitrogen ratio.	Apparent glutose.
Sept., 1925	*gm.*	*gm.*		
23–24 9 a.m.	19.6	75.6	3.85	
5 p.m.	21.9	80.0	3.64	
24–25 9 a.m.	19.2	69.5	3.63	
10 "	17.5 gm. glutose (8%) subcutaneously.			
5 p.m.	17.5 " " (8%) "			
25–26 9 a.m.	16.4	58.1	3.54	38.5
5 p.m.	16.2	57.2	3.53	7.0

The second subject, L. K., a diabetic, weighed 17.9 kilos. The R. Q. at 9.30 a.m. was 0.79. At 9.40, 35 gm. of glutose were taken. The R. Q. at 11.05 was 0.77 and at 12.20 it was still 0.77.

Effect of Glutose on Ketosis in Man.

Four subjects, two normal and two epileptic, were placed on a diet containing calories equal to basal requirements plus 50 per cent with a Ladd and Palmer (10) keto-antiketogenic ratio of approximately 4 : 1. After the subjects had been in a fairly even

ketosis for several days 50 to 70 gm. of glutose were added to the diet. In one epileptic the ketosis was halved, in another unchanged, while the two normal subjects showed a rise in acetone output on the glutose day. The nitrogen excretion was unaffected.

Action of Sodium Hydroxide on Glutose with Formation of Lactic Acid.

Glutose (100 gm.) in water (2 liters) was mixed with sodium hydroxide (100 gm.) and the closely stoppered mixture warmed to 60° for 3 days. Almost all of the glutose had disappeared by this time. The solution was made just acid to Congo red with sulfuric acid and the volatile acids removed by distillation. The amount of volatile acid was very small and was chiefly formic acid. Calculated as formic acid, it amounted to 0.51 gm. The concentrated residue was next extracted thoroughly with ether for 2 days. The ether extract in the experiments amounted to 41 and 43 gm. They contained much lactic acid which was readily separated as the zinc salt. 31 gm. of optically inactive zinc lactate were separated in one experiment and 29 in the other. The purity of the zinc lactate was controlled by analysis. The filtrate from the zinc lactate was treated with hydrogen sulfide to remove the zinc and on concentration each experiment gave a syrupy residue of about 10 gm. of unidentified hydroxy acids. The calcium salt contained 14.4 per cent calcium, but it was not found possible to identify the acids. They resembled complex products derived from glucose and fructose by Meisenheimer (6), and probably contained a variety of polyhydroxybutyric acids. Lactones were present in a very small amount, but a substance was noted which reduced ammoniacal silver solution in the cold and also Fehling's solution. Some slight evidence pointing to its being a hydroxylevulinic acid was obtained, but the identification was not satisfactory. Traces of succinic acid were also encountered, this being doubtless derived from the yeast used in the preparation of the glutose. The polyhydroxy acids remaining unextracted by ether resembled the mixture obtained by Meisenheimer from glucose and could not be satisfactorily identified. No saccharic acid could be detected. It may also be noted that experiments upon the action of excess of calcium hydroxide on glutose, under condition which would convert glucose into saccharic acid, failed to disclose the presence of any of the latter acid.

Formation of Methylglyoxal-bis-Phenylhydrazone from Glutose.

The formation of methylglyoxal from glutose was accomplished under similar conditions to those used by Neuberg and Oertel (11) in the case of other sugars. Glutose (4 gm.), and sodium carbonate (3 gm.) in water (200 cc.) were heated in a loosely corked flask on the water bath with phenylhydrazine (6.5 cc.). Flocks of osazone soon began to settle out from the hot solution. After 4 hours the solution was cooled and the sticky precipitation washed, first with 1 per cent sulfuric acid, and then with water, and finally a little methyl alcohol. The residue was crystallized from benzene and then again from 60 per cent alcohol. The substance was obtained in the form of fine yellow needles melting at 147–148°, and was identical with methylglyoxal-bis-phenylhydrazone prepared for comparison. On analysis the substance contained 22.4 per cent nitrogen, while the theoretical value is 22.2.

Formation of Methyliminazole from Glutose.

The action of zinc ammonium hydroxide on glutose was carried out under the conditions prescribed by Windaus (12). 50 gm. of glutose were mixed with two parts of concentrated ammonia, together with freshly precipitated zinc hydroxide obtained from 50 gm. of zinc sulfate. The clear solution soon began to deposit the light yellow zinc salt of methyliminazole and its quantity gradually increased during 2 months. The precipitate was then filtered off and dried. It weighed 13.2 gm., thus giving a yield fully comparable to that obtained by Windaus from other hexoses. The zinc salt was decomposed with hydrogen sulfide, filtered, neutralized with hydrochloric acid, and concentrated. The strong solution was then saturated with potassium carbonate and extracted with ether. Methyliminazole was readily obtained in good yield on distilling off the ether, and it was further characterized by its oxalate, crystallized from aqueous acetone, and melting at 205–206°, and its picrate, m.p. 159–160°, precipitated in ether solution and recrystallized from water. On analysis the picrate gave 22.7 per cent nitrogen compared with a calculated value of 22.6 per cent. These derivations were identical with those obtained by Windaus and Knoop from methyliminazole derived from glucose.

Action of Yeast upon Glutose in the Presence of Phosphates.

The experiments were made by allowing a mixture of yeast (15 gm.), glutose (20 gm.), crystallized sodium phosphate (10 gm.), and water (100 cc.) to stand at 22–25° for 12 hours. Then different preparations of yeast were used, active living yeast, a dry yeast preparation, and an acetone-yeast preparation. Appropriate blank experiments were carried out in which the glutose was either omitted or replaced by glucose. That the yeast preparations were active was shown by the fact that the dry yeast and acetone yeast preparations in the presence of glucose converted 90 per cent of the phosphate into organic hexose phosphate.

At the close of the digestions, the solutions were neutralized with ammonia and inorganic phosphate removed with magnesium acetate. After filtering the organic phosphate the filtrate was determined by evaporation with nitric acid and precipitation as magnesium ammonium phosphate in the usual way. In no case did the amount of organic phosphorus obtained from the glutose experiment exceed that of the corresponding blank experiment without glutose. The analytical data are, therefore, not reproduced but it may be mentioned that, while the "blank" and glutose results averaged 35 to 45 mg. of organic P_2O_5, in the case of glucose it amounted to 1.75 to 1.81 gm. It is clear, therefore, that glutose, unlike glucose, is not converted into a hexose phosphate by the action of yeast.

SUMMARY.

Glutose, a non-fermentable ketohexose, obtained by the regulated action of alkali upon glucose or fructose, has been investigated.

Glutose resembles glucose and its isomer fructose in the following respects. Experiments *in vitro* show that:

1. Sodium hydroxide converts glutose into hydroxy acids, principally optically inactive lactic acid, to about the same extent as glucose.

2. Glutose on warming with phenylhydrazine in feebly alkaline solution readily furnishes the osazone of methylglyoxal.

3. Zinc ammonium hydroxide converts glutose into methylimin-

azole, giving a yield equal to that obtainable from the fermentable hexoses.

Glutose differs from glucose and fructose in the following respects. Experiments *in vivo* show that:

4. The tolerance for glutose in the human organism is very low. With moderate doses it is excreted unchanged in the urine and by the bowel.

5. The excretion of glucose by human diabetics was not influenced by the administration of glutose.

6. Phlorhizinized dogs excrete glutose quantitatively unchanged. No extra glucose is formed.

7. Ketosis was variably affected by glutose.

8. The respiratory quotient in man is not changed after glutose consumption.

9. Insulin shock is not influenced by glutose.

10. It was found that yeast, under conditions which were favorable for the formation of a hexose phosphate produced no such ester from glutose.

It is suggested that this inability to form a hexose phosphate is related to the non-fermentability by yeast of glutose and its poor utilization by mammalian muscle. Glutose is, however, capable of attack by organisms of the coli type with production of gas.

Methods for the preparation and estimation of glutose are described.

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THE MINIMUM ENDOGENOUS NITROGEN METABOLISM.

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The experiment reported in this paper was designed to determine the absolute minimum total nitrogen excretion in the urine and stools, and the nature of this nitrogen, when a normal adult is maintained on a practically nitrogen-free diet with the total caloric requirements supplied in excess by carbohydrate and fat. Another purpose of this experiment was to demonstrate that this could be accomplished without harm to the subject even when the study was prolonged for an unusual length of time.

Some of the lowest 24 hour urinary total nitrogen excretions given in the literature (1–9) are shown in Table I in order to serve as a comparison with the data reported in this paper. The figure quoted from Sivén (9) is the lowest to be found. The work of Amato (10) lends the suggestion that this very low value may have been produced by the action of ingested alcohol, for Sivén states that his subject received 39.6 gm. of alcohol daily.

The first attempt to determine the nitrogen distribution in urines with low total nitrogen content was made in 1905 by Folin (1). He demonstrated that from such studies it is possible to obtain information concerning the mechanism of nitrogen metabolism. It would seem, from a review of the literature since 1905, that the value of Folin's experimental method has not been generally appreciated, for in only a few instances has his work been repeated. It is certain that his experiments were not done on subjects with nitrogen excretion at the absolute minimum. He recognized this fact and ventured predictions which made it highly desirable to repeat his experiments under conditions where

* The experimental work of this paper was done in 1923 at the New England Deaconess Hospital Laboratory.

an absolute minimum nitrogen excretion was a fact. In Table II is collected a summary of succeeding work of this nature carried out on humans. The results obtained by Robison are of greatest interest for they show even more contrast to the normal than those obtained by Folin, and in addition they corroborate the views which the latter had expressed. The use of the experimental method introduced by Folin affords a means for studying

TABLE I.

Studies on Minimum Nitrogen Excretion in the Urine.

Day of experiment.	Urine total nitrogen.	Body weight.	Nitrogen per kilo.	Author.
	gm.	*kg.*	*gm.*	
10	3.8	64.0	0.0594	Folin (1).
4	3.76	69.7	0.0539	Landergren (2).
5	3.5	70.5	0.0497	Folin (1).
4	3.04	62.4	0.0487	Landergren (2).
5	2.8	55.7	0.0485	Folin (1).
8	3.12	63.5	0.0480	Klemperer (3).
7	3.34	71.3	0.0468	Landergren (2).
7	2.42	57.5	0.0421	Roehl (4).
12	2.6	64.0	0.0406	Folin (1).
8	2.51	65.0	0.0395	Klemperer (3).
19	2.98	76.2	0.0391	Thomas (5).
9	2.25	61.4	0.0366	Graham and Poulton (6).
11	1.99	57.8	0.0344	Robison (7).
6	2.01	88.0	0.0319	Klerker (8).
7*	1.84	58.0	0.0317	Sivén (9).
24	1.58	65.3	0.0242	Smith (this paper).

* This subject had previous to the last 7 days of this experiment been on a moderate nitrogen restriction. On the 2nd day of the last 7 day period the urine nitrogen was 1.78 gm. This appears to be an error, for the day before and the day after the urine nitrogen was 2.83 and 2.22 gm. respectively.

the factors which are concerned in the nitrogen economy of the body, because with diets such as he used their action becomes more apparent. Some of them will be briefly mentioned.

It is well known that the endogenous nitrogen metabolism is spared by carbohydrate and fat. Therefore it is important to supply the caloric requirements of the body with carbohydrate and fat in excess when a study of the minimum endogenous nitrogen metabolism is to be made.

An adequate supply of the essential amino acids as well as vitamins is necessary when nitrogen restriction is to be prolonged for periods over 2 or 3 weeks.

The influence of inorganic salts upon the nitrogen metabolism is adequately demonstrated by the work of Asada (11), McCollum and Hoagland (12), Berg (13), Jansen, Biehler, and Legène (14), Richards, Godden, and Husband (15), Grafe and Schlapfer (16), Abderhalden and Hirsch (17), Terroine, Fleuret, and Stricker (18), and others. This is a very fertile field for further study, for the opinions of these workers are at considerable variance.

In the experiment of this paper these factors could not be studied, for much of the work was taken up with an attempt to arrange a palatable basic diet which could be continued over extended periods. Moreover this was mainly an attempt to reduce the endogenous nitrogen metabolism to an irreducible minimum, and to demonstrate that no harm was incurred by the subject therefrom.

EXPERIMENTAL.

The subject was a healthy and active medical student of 28 years, weighing 64.75 kilos and measuring 151.8 cm. in height. His usual dietary contained on the average 75 gm. of protein daily and sufficient calories to have maintained his weight constantly during the preceding year at the above level. His activities were varied and he was both mentally and physically vigorous. His faithfulness to adherence to diet and accurate collection of urine and stool specimens was beyond question.

It was planned to feed the subject exclusively upon a carbohydrate diet which would be as nitrogen-free as possible. This purpose was soon thwarted by the extreme nausea developed upon such a high caloric and sweet diet. It was not until after 12 days that a diet could be planned which was palatable and minimal in nitrogen content and contained a caloric excess. After much experimenting a bread was made out of corn-starch, India gum, yeast, sugar, lard, and salt. The palatability was increased by the inclusion of foods low in nitrogen content, such as lettuce, cucumbers, tomatoes, jam, peaches, pears, and heavily sweetened lemonade. With this diet the subject did not complain of nausea and it was possible to give as high as 4000 calories without difficulty. The food was not analyzed for nitrogen, the values being calculated from the average analyses of Atwater and Bryant (20).

The subject was first observed over a preliminary week with unrestricted diet. The analyses of blood, urine, and stools are shown in Table III. Martin (21) strength tests and basal metabolism determinations were carried

TABLE II.

Day of experiment.	Urine nitrogen as:								As per cent of total N.							Remarks.	Author.
	Total.	Urea.	NH_3	Creatinine.	Uric acid.	Amino acids.	Purine bases.	Rest.	Urea.	NH_3	Creatinine.	Uric acid.	Amino acids.	Purine bases.	Rest.		
	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	per cent	per cent	per cent	per cent	per cent	per cent	per cent		
	15.80	13.90	0.54	0.43	0.11			0.88	87.7	3.3	2.7	0.7			5.6	Subject E. S. A. Control day.	Folin (1).
7	2.80	1.70	0.28	0.41	0.08			0.32	60.7	10.0	14.0	3.0			11.6	" "	"
7	3.50	2.10	0.27	0.50	0.14			0.52	59.3	7.8	14.1	4.0			14.8	" Aug. H.	"
5	2.81	1.53	0.07	0.70					54.4	2.5	24.9					43.4 calories per kilo.	Klerker (8).
9	3.65	1.78	0.41	0.68	0.18			0.60	48.8	11.2	18.6	4.9			16.5	43.4 " " "	Klerker.
14	5.32	3.21	0.64	0.61	0.08	0.19	0.01	0.48	60.4	12.0	11.5	1.6	3.6	0.1	9.0	Subject 70 kilo man. Diet, carbohydrate 0 per cent, fat 100 per cent.	Zeller (19).
4	2.99	1.49	0.34	0.63	0.16	0.24	0.02	0.07	49.7	11.4	21.1	5.4	7.8	0.6	2.2	Same subject. Diet, carbohydrate 100 per cent, fat 0 per cent.	"
19	5.53	2.86	0.85	0.67	0.11	0.30	0.02	0.53	51.7	15.4	12.1	1.9	5.4	0.3	9.6	Subject 80 kilo man. Diet, carbohydrate 0 per cent, fat 100 per cent.	"
3	3.52	1.40	0.30	0.66	0.18	0.28	0.02	0.60	39.7	8.5	18.8	5.6	7.9	0.4	17.1	Same subject. Diet, carbohydrate 100 per cent, fat 0 per cent.	"

9	2.25	1.05	0.34	0.60	0.07			0.19	46.5	15.2	26.6	3.2			8.4	Subject 61.4 kilo man. Nitrogen intake 0.91 gm.	Graham and Poulton (6).
11	2.13	1.08	0.21	0.43	0.07	0.05		0.29	50.7	9.9	20.2	3.3	2.3		13.6	Subject 60.5 kilo man. Nitrogen intake 0.33 gm.	Robison (7).
11	1.99	0.74	0.35	0.50	0.07	0.04		0.29	37.2	17.6	25.1	3.5	2.1		14.5	Subject 57.8 kilo man. Nitrogen intake 0.33 gm.	Robison.
24	1.58	0.35	0.23	0.56	0.11			0.33	22.1	14.5	35.4	7.0			21.0	Subject 65.3 kilo man. Nitrogen intake 0.33 gm.	Smith (this paper).

TABLE III.

Date, 1923	Volume, 24 hrs.	Urine analysis. Nitrogen as: Total.	Urea.	NH_3	Creatinine.	Uric acid.	Rest.	As per cent of total nitrogen. Urea.	NH_3	Urea + NH_3.	Creatinine.	Uric acid.	Rest.	Sulfur as S. Total.	Inorganic + ethereal.	Inorganic.	Ethereal.	Organic.	As per cent of total sulfur. Inorganic.	Ethereal.	Organic.	Phosphate as P.	NaCl
Mar.	*cc.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*per cent*	*per cent*	*per cent*	*gm.*	*gm.*
26	1340	14.25		0.55	0.67	0.15			3.9		5.6	1.1										0.99	5.91
27	1005	13.10		0.44	0.67	0.17			3.4		5.1	1.3										0.91	8.04
28	1105	12.33		0.51	0.70	0.15			4.1		5.7	1.2										0.66	11.06
29	1410	12.47		0.54	0.74	0.13			4.3		6.0	1.0										0.78	11.74
30	1425	9.32	7.62	0.41	0.63	0.11	0.55	81.8	4.4	86.2	6.7	1.2		0.61	0.56			0.057			9.3	0.63	13.97
31	1840	10.93			0.66	0.15					6.0	1.4		0.70	0.63			0.073			10.4	0.70	18.03
Apr.																							
1	1510	11.78	9.61	0.54	0.66	0.17	0.80	81.6	4.6	86.2	5.6	1.4	5.9	0.83	0.77			0.061			7.3	0.73	11.10
2	1395	7.28			0.66	0.08					9.1	1.1		0.74	0.66			0.083			11.2	0.59	2.57
3	495	4.56	3.02	0.70	0.62	0.10	0.12	66.2	15.4	81.6	13.6	2.2	2.6	0.28	0.22			0.060			21.4	0.74	0.62
4	330	3.90			0.63	0.12					16.2	3.1		0.25	0.18	0.16	0.020	0.070	64.0	8.0	28.0	0.31	0.30
5	1150	4.69				0.15						3.2		0.25	0.16	0.16	0.005	0.087	64.0	2.0	34.8		0.35
6	1420	4.67				0.13						2.8		0.34	0.26	0.23	0.028	0.081	67.7	8.2	23.8		0.21
7	1330	3.99				0.17						4.3		0.32	0.23	0.21	0.017	0.087	65.6	5.3	27.2		0.15
8	1015	3.78				0.21						5.6		0.29	0.20	0.19	0.012	0.084	65.5	4.1	29.0		0.23
9	1110	3.23				0.19						5.9		0.25	0.17	0.16	0.010	0.074	64.0	4.0	29.6		0.67
10	1295	3.69				0.21						5.7											0.26
11	700	3.63																					0.20
12	940	4.29				0.12						2.8											0.42
13	965	3.35																					0.31
14	675	2.88																					0.36
15	850	2.67			0.56	0.15					21.0	5.6		0.21	0.14	0.13	0.016	0.062	61.9	9.6	29.5	0.22	0.42
16	415	2.37	1.08	0.31	0.56	0.10	0.32	45.6	13.1	58.7	23.6	4.2	13.5	0.24	0.18	0.15	0.035	0.061	62.5	15.5	25.4	0.12	0.30
17	470	2.18	0.85	0.34	0.59	0.12	0.28	39.0	15.6	54.6	27.1	5.5	12.8	0.24	0.18	0.15	0.027	0.062	62.5	11.3	25.8	0.15	0.46
18	415	2.10	0.69	0.34	0.56	0.11	0.40	33.0	16.2	49.2	27.0	5.2	18.6	0.23	0.17	0.15	0.018	0.059	65.2	7.8	25.7	0.09	0.62
19	905	2.13	0.78	0.36	0.56	0.12	0.31	36.0	16.9	52.9	26.3	5.6	15.2	0.22	0.16	0.13	0.021	0.065	59.1	9.5	29.5	0.11	1.12
20	550	2.16	0.66	0.37	0.56	0.16	0.41	30.6	17.1	47.7	22.9	7.4	22.0	0.21	0.16	0.12	0.039	0.059	57.1	18.6	28.1	0.15	1.64
21	430	2.09	0.60	0.36	0.60	0.12	0.41	28.7	17.2	45.9	28.7	5.7	19.7	0.29	0.23	0.20	0.026	0.061	69.0	9.0	21.0	0.07	4.90
22	540	2.01	0.48	0.34	0.58	0.15	0.46	23.9	16.9	40.8	28.9	7.5	22.8	0.29	0.24			0.052			17.9	0.04	11.64
23	1500	1.86	0.37	0.29	0.59	0.14	0.47	20.0	15.6	35.6	31.7	7.5	25.2	0.29	0.23	0.21	0.015	0.058	72.4	5.2	20.0	0.05	15.20
24	985	1.68	0.29	0.16	0.56	0.13	0.54	17.3	9.5	26.8	33.3	7.7	32.2	0.23	0.17	0.15	0.024	0.058	65.2	10.4	25.2	0.09	8.20
25	970	1.58	0.35	0.23	0.56	0.11	0.33	22.1	14.5	36.6	35.4	7.0	21.0	0.19	0.13	0.11	0.019	0.064	57.9	10.0	33.7	0.10	5.40
26*	150	0.52	0.09	0.06	0.18	0.04	0.15	17.3	11.5	28.8	34.6	7.7	28.9	0.05	0.03	0.02	0.007	0.020	40.0	14.0	40.0	0.01	1.56
26†	680	3.10	1.84	0.13	0.41	0.09	0.63	60.0	4.2	64.2	13.2	2.9	19.7	0.16	0.11	0.11	0.008	0.050	68.8	5.0	31.3	0.14	5.00
27	1595	8.82	6.65	0.36	0.59	0.12	1.10	75.4	4.1	79.5	6.7	1.4	12.4	0.41	0.28	0.25	0.034	0.126	61.0	8.3	30.7	0.22	10.80
28	1640	9.60	7.90	0.50	0.57	0.14	0.49	82.3	5.2	87.5	5.9	1.5	5.1	0.55	0.42	0.39	0.028	0.126	70.9	5.1	22.9	0.32	17.60

* 7.00 a.m.–3.00 p.m.

† 3.00 p.m.–7.00 a.m.

TABLE III.

Phosphate as P.	NaCl	Food intake. Fluid.	Total calories.	Carbohydrate.	Fat.	Protein.	Nitrogen.	Feces nitrogen.	Nitrogen balance.	Body weight, morning.	Blood. Non-protein nitrogen. Plasma.	Non-protein nitrogen. Whole blood.	Uric acid. Plasma.	Uric acid. Whole blood.	NaCl Plasma.	NaCl Whole blood.	P Plasma.	P Whole blood.	Sugar. Plasma.	Sugar. Whole blood.	Cell volume.
gm.	*gm.*	*cc.*	*cals.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*kg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*per cent*
0.99	5.91	Unrestricted.									27	35	4.4	3.8	577	451	6.1	6.3		102	46.3
0.91	8.04	"						1.03													
0.66	11.06	"						1.03													
0.78	11.74	"						1.03													
0.63	13.97	"						1.03													
0.70	18.03	"						1.03													
0.73	11.10	"						1.03													
0.59	2.57	1500	2485	527	41.7	0.35	0.06	0.85	−8.07	64.75											
0.74	0.62	1500	2515	553	33.5	0.35	0.06	0.85	−5.35	64.41											
0.31	0.30	1500	2249	521	18.2	0.35	0.06	0.85	−4.69	64.18											
	0.35	900	1436	336	10.2	0.12	0.02	0.85	−5.52	64.64											
	0.21	1600	2368	478	48.2	5.55	0.89	0.85	−4.63	64.00											
	0.15	2300	3328	617	94.0	3.58	0.56	0.85	−4.28	64.18											
	0.23	1575	2460	416	87.0	3.27	0.52	0.73	−3.99	63.73											
	0.67	1000	2997	640	47.0	3.46	0.55	0.73	−3.41	64.00											
	0.26	1500	1974	355	60.0	3.58	0.56	0.73	−3.86	63.73											
	0.20	1000	1630	292	49.0	5.20	0.83	0.73	−3.50	63.73											
	0.42	1500	3151	596	83.0	5.10	0.83	0.73	−4.19	63.18											
	0.31	1400	2819	609	40.0	6.55	1.05	0.73	−3.03												
	0.36	1500	3426	719	58.0	6.36	1.02	0.73	−2.59	63.05	17	25	5.5	4.0	575	460	7.2	8.1		100	44.8
0.22	0.42	1500	3513	655	96.0	6.48	1.04	0.73	−2.36	63.73											
0.12	0.30	1500	3425	608	107.0	6.52	1.04	0.84	−2.17	64.41											
0.15	0.46	1500	3352	596	105.0	4.88	0.78	0.84	−2.24	63.73											
0.09	0.62	1500	3141	569	93.0	6.07	0.97	0.84	−1.97	64.64											
0.11	1.12	1500	3095	607	71.0	5.97	0.96	0.84	−2.01	64.64											
0.15	1.64	1600	3224	655	64.0	6.89	1.10	0.84	−1.90	64.64											
0.07	4.90	2350	3983	634	158.0	6.13	0.98	0.87	−1.93	64.64	15	24	5.4	4.1	604	481	5.9	11.1		104	43.6
0.04	11.64	2100	4107	643	169.0	3.89	0.62	0.87	−2.26	65.10											
0.05	15.20	1600	4121	668	160.0	2.97	0.48	0.87	−1.86	65.55											
0.09	8.20	1400	3157	561	101.0	2.08	0.33	0.91	−2.26	65.32	13	21	2.9	3.0					102	118	
0.10	5.40	1500	3157	561	101.0	2.08	0.33	0.91	−2.16	65.32											
0.01	1.56	400	837	156	23.0	0.86	0.13			65.55											
0.14	5.00	2000	565	33	32.0	37.00	5.92														
0.22	10.80	Unrestricted.								65.55											
0.32	17.60	"																			

out at intervals. The chemical methods used are given in the Appendix together with data relative to the diet.

On the 25th day of the low nitrogen diet the subject became so alarmed at his extremely low nitrogen excretion of the day before that he lost his courage, and in the middle of the day (3.00 p.m.) decided that he could continue no longer. From this point he had an unrestricted diet and the urine nitrogen distribution shows a rapid return to normal in 2½ days.

Consideration of Data.

While the lowest total 24 hour urinary nitrogen excretion on record for a normal adult was attained, it is certain that the absolute minimum was not reached because the food was not entirely nitrogen-free. 0.33 gm. of the urinary nitrogen for the last 2 days must be considered as exogenous nitrogen. It is approximately accurate to consider this nitrogen as excreted in the form of urea or ammonia.

An inspection of Table III shows the greatest contrast to the usual in nitrogen distribution that has ever been found in the human. This contrast bears with it considerable significance and it is possible to develop a rather interesting and suggestive theory from a comparison of the urea plus ammonia nitrogen and creatinine nitrogen values. Before this can be done certain premises will have to be reviewed briefly.

It is generally considered that ammonia is formed at the expense of urea and that they have a common source. Therefore their sum will be considered in the nitrogen balance calculations to follow. The metabolic products of food nitrogen will be considered as consisting entirely of amino acids. Creatine will be considered as the precursor of creatinine, and arginine of creatine. For an extended review of this latter premise see the monograph of Hunter (22). Since arginine has four nitrogen atoms and creatine and creatinine each have three, it follows that for every molecule of creatine formed, one nitrogen atom from the amino group of the arginine molecule will be discarded. It will be assumed that this group will be excreted as urea or ammonia. If creatinine is formed from creatine, and the supply of the latter is constantly replenished from arginine molecules, then it should be possible to determine how much nitrogen is excreted in the urine in the form of urea or ammonia representing the discarded amino group from the arginine molecule, if the creatinine nitrogen is

divided by three. A final premise is that the food nitrogen is entirely absorbed and that the nitrogen balance as far as the food is concerned may be determined by subtracting the urea plus ammonia nitrogen from the food nitrogen. This latter point cannot be argued here but there seems to be sufficient evidence to justify such a conclusion, and the author believes it to be true under the conditions of this experiment.

In Table IV the food nitrogen is balanced against the urea plus ammonia nitrogen for the last 10 days of the experiment. There is a small negative balance on all days except 2. This negative balance must represent the products of protein or amino acid loss

TABLE IV.

Date, 1923.	Food nitrogen.	Urine nitrogen as:			Nitrogen balance.	Creatinine nitrogen divided by 3.
		Urea.	Ammonia.	Urea plus ammonia.		
Apr.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
16	1.04	1.08	0.31	1.39	−0.35	0.19
17	0.78	0.85	0.34	1.19	−0.41	0.20
18	0.97	0.69	0.34	1.03	−0.06	0.19
19	0.96	0.78	0.36	1.14	−0.18	0.19
20	1.10	0.66	0.37	1.03	+0.07	0.19
21	0.98	0.60	0.36	0.96	+0.02	0.20
22	0.62	0.48	0.34	0.82	−0.20	0.19
23	0.48	0.37	0.29	0.66	−0.18	0.20
24	0.33	0.29	0.16	0.45	−0.12	0.19
25	0.33	0.35	0.23	0.58	−0.25	0.19

from the body. The theory which the author wishes to propose is that this loss represents chiefly the discarded amino groups from arginine molecules which are used for the formation of creatine. In the last column of Table IV are the respective creatinine excretions divided by three. The agreement is very close for the last 4 days of the experiment which represent the nearest approach to the absolute minimum endogenous nitrogen metabolism.

This theory reduces the nitrogenous metabolism of the muscles to a very simple conception. As long as the energy requirements of the muscle cells are supplied by carbohydrate and fat, their chief nitrogen demands consist of the replenishment of a small

amount of creatine from the amino acid arginine. The protein or amino acid requirements for glandular metabolism would seem to be almost negligible under conditions where the energy is supplied by carbohydrate and fat. Muscle cells and particularly gland cells require a certain amount of purine nitrogen but either this is a very small quantity or the body cells have marked powers to conserve this form of nitrogen or to resynthesize the products of purine catabolism.

The "rest" nitrogen forms a relatively large proportion of the total nitrogen. This designation implies a significance which is out of proportion to the actual importance of this fraction of the total nitrogen. Kohn (23) has made a very complete analysis of this nitrogen distribution of a human urine, and cites references to the work of others on the less commonly determined nitrogen constituents. The following table shows the complete nitrogen distribution in the urine which she analyzed. In this

	Total nitrogen.	
	per cent	*gm.*
Urea	82.9	4.352
Ammonia	8.6	0.451
Uric acid	1.2	0.063
Creatinine	4.5	0.236
Amino acids	0.3	0.016
Purine bases	0.3	0.016
Oxyproteic acids	1.5	0.079
Hippuric acid	2.9	0.152
	102.2	5.365

case hippuric acid formed over half of the nitrogen which is usually undetermined. It would seem from the above figures that serious and irreplaceable nitrogen losses are not involved in the rest nitrogen portion.

The inorganic metabolism will not be considered because the inorganic content of the diet was not controlled. It should be mentioned, however, that the rise in urine chloride towards the end of the experimental period was due to increased sodium chloride ingestion. It would seem that this was a factor in the final reduction of the total nitrogen of the urine.

The blood total non-protein nitrogen figures assumed an unusually low level. It is to be regretted that no blood urea determinations were done, for it is possible that only a trace would have been found.

In Table V are arranged, from Thomas (5) values for stool nitrogen obtained from adults maintained on nitrogen-free or

TABLE V.

Daily Stool Nitrogen on Low Protein Diets.

Food nitrogen.	Stool nitrogen.	Author.
gm.	*gm.*	
1.36	1.39	Rubner (25).
0.29	0.54	Rieder (26).
0.37	0.87	"
0.30	0.78	"
3.00	1.26	Sivén (9).
4.00	1.23	"
1.00	0.75	Landergren (2).
2.40	1.47	"
2.00	1.02	"
2.42	1.33	"
2.19	1.28	"
0.27	1.14	"
0.06	0.96	"
1.65	0.58	"
0.24	0.46	"
1.65	0.22	"
3.50	1.75	Klerker (8).
0.14	0.65	Tigerstedt (27).
0.22	1.50	Renvall (28).
0.31	1.52	"
0.00	0.45	Thomas (5).
0.00	0.97	"
1.04	0.73	Smith (this paper).
0.33	0.91	" " "

nearly nitrogen-free diets. The rather marked variation would seem to argue against the excretion of food nitrogen residue, and more for variations in intestinal secretions and cellular débris. The work of Voit (24) lends support to such an argument. In Table III the difference between the stool nitrogen on an unrestricted diet and that when the protein restriction was the

greatest is seen to be so slight that the presence of food residue nitrogen in the stools is questionable. It is therefore more accurate, in figuring the total nitrogen balance, to consider the stool nitrogen as body nitrogen.

The total nitrogen balance averaged 3.34 gm. on the negative side per day throughout the experiment. For the total experimental period the nitrogen loss was 80.08 gm. The fact that the subject gained slightly in weight, led a very active life, and showed a rapid return to normal nitrogen distribution in the urine after

TABLE VI.

Strength Tests (Kilos).

	Mar. 24.	Mar. 31.	Apr. 6.	Apr. 12.	Apr. 20.	Apr. 27.	May 8.	May 16.	June 14.
Pectoral left	38	35	36	36	35	34	36	31	31
Pectoral right	38	35	38	36	37	35	38	35	36
Wrist flexors left	23	20	22	22	22	23	23	22	23
Wrist flexors right	22	20	24	24	22	21	20	23	21
Forearm flexors left	31	35	30	33	31	29	32	28	30
Forearm flexors right	33.5	33	30	33	31	30	33	28	27
Thigh abductors left	19.5	19	18	17	18	17	17	17	17
Thigh abductors right	21.5	20	21	20	19	19	20	18	20
Thigh adductors left	20	16	20	17	18	17	12	16	17
Thigh adductors right	20	17	19	19	19	17	15	16	16
Total	266.5	250	258	257	252	242	246	234	238
" strength	1506	1413	1458	1452	1424	1367	1390	1322	1345

the resumption of an unrestricted diet, argues that very little if any harm could have been incurred by this prolonged negative nitrogen balance. It may be of interest to mention that the author has kept a nephritic subject in negative nitrogen balance for a period of 8 months without apparent harm.

In Table VI are arranged the data of the strength tests. The total strength is obtained by multiplying the sum of the pulls of the five paired groups of muscles by 5.65 (see Martin (21)). The total strength remained quite constant until the end of the

experiment and then there was a sudden drop of 5 per cent in the total strength. For the reason that even after nearly 2 months from the close of the experiment the strength had not been regained it can be argued that there was no real loss of strength due to nitrogen restriction. The subject was eager to carry out the experiment at the beginning but was not so enthusiastic at the end,

TABLE VII.

Basal Metabolism Tests.

Date, 1923.	Naked weight.	Pulse.	Urine nitrogen per hr.	Oxygen per min.	CO_2 per min.	R. Q.	Basal metabolism found.	Basal metabolism predicted (Harris and Benedict).	Variation from standard.
	kg.		*gm.*	*cc.*	*cc.*		*cals.*	*cals.*	*per cent*
Mar.									
24	65.5	64	0.792	211	170	0.81	1462	1648	−11
30	64.8	62	0.363	199	163	0.82	1383	1639	−16
Apr.									
14	62.2	65		198	166	0.84	1383	1603	−14
24	64.1	71		210	200	0.95	1507	1629	−7

and this fact may be reflected in the vigor with which he carried out his strength tests during and after the experiment.

The metabolism data are given in Table VII.

CONCLUSIONS.

It is believed that the absolute minimum endogenous nitrogen metabolism was obtained in the subject of this experiment. The urine does not accurately represent this true minimum because the diet contained a small amount of nitrogen which appears in the urine chiefly as urea and ammonia. If this food nitrogen is subtracted from the urea plus ammonia nitrogen of the urine an approximate picture of the true minimum endogenous nitrogen metabolism is obtained. The "rest" nitrogen fraction must be left out of the picture for the present.

This prolonged protein restriction and continued negative nitrogen balance was not accompanied by any objective deleterious effects on the subject.

SUMMARY.

1. The purpose of this experiment was to determine the absolute minimum total nitrogen excretion in the urine which could be attained in a normal adult whose caloric requirements were supplied in excess by carbohydrate and fat only. As small a quantity of protein as possible, without making the diet intolerable, was given.

2. It was desired to prove that such a diet could be prolonged without harm to the subject. This expectation was fulfilled for a period of 24 days.

3. The subject was in negative nitrogen balance throughout the experiment. The total nitrogen lost in the 24 days was 80.08 gm.; the average daily loss was 3.34 gm.

The author wishes to acknowledge the fact that his interest in this phase of metabolism was stimulated by Dr. F. M. Allen, who demonstrated to him the value of rigid nitrogen restriction in cases with nitrogen retention. To Dr. Reginald Fitz for the strength tests the author is indebted. For laboratory facilities and encouragement the kindness of Dr. E. P. Joslin is acknowledged.

APPENDIX.

Chemical Methods Used.

1. Urine Total Nitrogen.—Urine was diluted until the nitrogen content was approximately that of a Folin-Wu blood filtrate. 5 cc. were then digested according to the method for blood non-protein nitrogen of Folin and Wu. The urine for the last 10 days was preserved with chloroform in an ice box and later the total nitrogen was again determined in duplicate by the original Kjeldahl method. The data by the two methods varies by about 2 per cent. The macro Kjeldahl results are given in Table III.

2. Stool Total Nitrogen.—The stools were collected in a closed jar for each period. Bacterial action was inhibited with formaldehyde, and the contents of the jar were made acid with hydrochloric acid. The stools were dried in a warm air oven at 90–95°C. After thorough pulverization of the dried residue, duplicate portions were taken for nitrogen determinations by the macro Kjeldahl method.

3. Urea Nitrogen.—The aeration method of Van Slyke and Cullen was used (29).

4. Ammonia Nitrogen.—10 cc. of urine were made alkaline with 2 gm. of potassium carbonate. 10 gm. of potassium oxalate were added and the ammonia aerated into tenth normal hydrochloric acid (30).

5. Creatinine Nitrogen.—The colorimetric method of Folin using a creatinine zinc chloride standard was employed (30).

6. Uric Acid Nitrogen.—Essentially the method described by Folin in his Manual was used. The writer has found that in normal urines the silver lactate precipitation is not necessary. With the exception of the omission of this step the method was followed in every detail.

7. Sulfate Determinations.—The method used is that of Fiske (31).

8. Phosphates in Urine and Blood.—Brigg's modification of the Bell and Doisy colorimetric method was used (32).

9. Urine Chlorides.—Standard silver nitrate solution was added to 10 cc. of urine. After strongly acidifying with nitric acid, and the addition of ferric ammonium sulfate crystals, the excess silver nitrate was determined with standard potassium sulfocyanate solution. The titration was done without removing the silver chloride.

10. Blood Non-Protein Nitrogen and Blood Sugar.—The method of Folin and Wu was used.

11. Blood Uric Acid.—See Folin's revised method for determination of blood uric acid (33).

12. Blood Chlorides.—The blood was taken under oil, potassium oxalate being used as an anticoagulant. Chlorides were determined by a method devised by the writer (34).

Diet for Last 3 Days of Experiment.

	Protein.	Fat.	Carbohydrate.	Calories.
	gm.	*gm.*	*gm.*	
7.30 a.m.				
Grapefruit, 100 gm	0.60	0.10	12.20	52.10
Sugar, 50 gm			50.00	200.00
Coffee (weak), 150 cc.				
Cream* (homogenized butter), 10 gm	0.04	3.40		30.76
Sugar, 40 gm			40.00	160.00
Bread† (from corn-starch)	0.02	2.81	53.58	239.69
Butter, 20 gm	0.20	16.50		153.80
12.00 noon.				
Canned pineapple, 50 gm	0.20	0.35	18.20	76.75
Bread (from corn-starch)	0.02	4.15	79.13	353.95
Butter, 40 gm	0.40	34.00		307.60
4.00 p.m.				
Lemonade (50 per cent sugar), 200 cc.			100.00	400.00

Diet for Last 3 Days of Experiment—Concluded.

	Protein.	Fat.	Carbohydrate.	Calories.
	gm.	*gm.*	*gm.*	
6.00 p.m.				
Salad.				
Lettuce, 15 gm	0.18	0.06	0.04	2.90
Cucumbers, 25 gm	0.20	0.05	0.80	4.45
Salad dressing‡		20.00	3.00	192.00
Bread (from corn-starch)	0.02	2.81	53.58	239.69
Butter, 20 gm	0.20	16.50		153.80
9.00 p.m.				
Lemonade (50 per cent sugar), 300 cc.			150.00	600.00
	2.08	100.73	560.53	3167.49

* Synthetic cream. Salt-free butter, with the aid of gum acacia, was homogenized by the Walker Gordon Company into a 40 per cent cream.

† Recipe for corn-starch bread.

Dissolve one-half yeast cake in 36 cc. of luke warm water. In a 3 quart pan with cover add the following: lard, 10 gm.; butter, 10 gm.; sugar, 15 gm.; salt, 2 to 4 gm.; boiling water, 350 cc. Stir until butter and lard are thoroughly dissolved. Measure 375 gm. of corn-starch and 40 gm. of India gum into a large paper bag. Shake until thoroughly mixed. After the lard and butter mixture has cooled so that it is luke warm, the yeast is added to it and mixed well. Then add the corn-starch mixture, in small portions, with vigorous stirring. After thorough mixing the pan is covered and set aside in a warm place for about 12 hours. The dough should not be kneaded after rising, but placed directly in the pan in which it is to be baked. It requires a hot oven for about 45 minutes. One loaf contains: carbohydrate, 352.5 gm.; fat, 18.5 gm.; protein, 0.1 gm.; calories, 1567.9. The bread is quite palatable if eaten fresh. It soon (within 8 hours) becomes gummy and unpalatable. Further work is now in progress to see if this can be avoided. Gum acacia seems to be a better gluten substitute than India gum.

‡ Recipe for salad dressing. The following mixture is homogenized by vigorous stirring: olive oil, 20 gm.; vinegar, 15 gm.; sodium chloride, 2 gm.; mustard, 2 gm.; pepper, trace; paprika, trace. The food value is divided between: fat, 20 gm.; carbohydrate, 3 gm.; calories, 192.

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DISTRIBUTION OF UNSATURATED FATTY ACIDS IN TISSUES.

I. BEEF HEART MUSCLE.

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The fat of the body stores must in the nature of the case be resistant to oxidation under the conditions of storage but the same fat must later be burned under conditions apparently not very different from those under which it is stored. It is therefore of the greatest importance in the study of the metabolism of fat to know what the changes are that render it readily combustible under the exacting conditions consistent with life in the animal body. The changes undergone by the fats, or more strictly by the fatty acids which are their essential constituents, may be of two kinds—changes in the fatty acids themselves or changes in the nature of the combinations in which they exist. As stored, the fatty acids are practically completely in combination as fat—mixtures of triglycerides. In the tissues and organs available data indicate that they are combined in various forms—phospholipids, cholesterol esters, etc. As stored, the fatty acids are largely either saturated or of a low degree of unsaturation and although recent work such as that of Eckstein (1) has shown that small percentages of more unsaturated acids may be present (in human fat about 0.5 per cent of the total fatty acid as two double bond acid, half as much of four double bond acid, and traces of a three double bond acid) the general statement probably holds true. Fattening experiments on hogs, such as those reported by Ellis and Hankins (2) show that oleic acid is formed in large amount by synthesis from carbohydrate while the linolic acid found was referable to the fat of the food. In the tissues and organs there are found in addition to oleic acid, which is the main unsaturated acid in both tissue and

stored fat, larger amounts of the more unsaturated acids up to and including arachidonic acid with four double bonds. The only tissue on which any considerable quantitative data are available is the liver which has been the subject of investigation by Leathes and his colleagues. Their observations, which have been referred to many times in work from this laboratory, may be summarized as follows: The liver fatty acids differ from those of the fat stores in being considerably more unsaturated, having an iodine absorption value of about 90 as compared with 60 to 70 for the stored fatty acids, while a relatively large proportion of them are in combination as phospholipids—lecithin, cephalin, etc. Evidence is presented to show that the liver desaturates the fatty acids and is probably instrumental in transforming part at least of the ordinary fat into phospholipids. Significant in in connection with the present work is the fact that Hartley (3) found in the liver three unusual acids—an oleic acid with a double bond in a different position (6 to 7 from the methyl) from that in ordinary oleic acid (9 to 10), a C_{18} acid with double bonds in both these positions, and also a C_{20} carbon acid with four double bonds (arachidonic) which Levene (4) has located definitely in the lecithin. Recent work by Wesson (5) has shown that arachidonic acid is widely distributed in the tissues and organs of animals.

Data on the nature and manner of combinations of the fatty acids of blood have been presented (6) which go to show that in plasma, most of the unsaturated fatty acids are in combination as cholesterol esters, the phospholipid fractions containing relatively much less, while the fat apparently contains more of the unsaturated acids than the phospholipid, but less than the cholesterol esters. In experimentally anemic animals (7) the lecithin fraction and the fat fraction of the blood lipids contain increased amounts of unsaturated acids, while the cholesterol esters are unaffected.

Erlandsen (8) made an extensive study of the lipids[1] of beef heart

[1] The biochemistry of the fatty substances has been needlessly complicated by the use of several terms for the group and subgroups. The use of still another set of terms may not be the obvious cure for the condition but the term lipid for the whole group has the sanction of the International Congress of Pure and Applied Chemistry and so is in a fair way to general adoption. The names for the subgroups, for example phospholipid, are self-explanatory. A classification making use of the new terms is given in detail in an earlier publication (9).

muscle particularly the phospholipids. His attention was, however, mainly directed to the phosphorus and nitrogen of these compounds and his work contains very little reference to the fatty acids. He found that the iodine value of the fatty acids from his cuorin (probably cephalin) was 130 while those from lecithin gave a value of 110 which he states must be low, since the lecithin itself gives a value of 100.

Constantino (10) found that the iodine number of the total fatty acids of smooth and heart muscle of beef was the same (about 130) while the acids of striated muscle had a lower value (about 96), due probably to the fact that striated muscle contains more stored fat.

Such data as are available regarding the occurrence of the fatty acids in brain and nerve tissue go to show that they are present in a high degree of unsaturation and combined in a number of forms most of which are chemically not well defined. Levene (11) found in brain cephalin arachidonic and other unsaturated acids in addition to oleic acid. MacArthur (12) reported 10 per cent of cephalinic and 5 per cent of chrysanodonic acid in brain cephalin in addition to oleic and stearic acids.

The peculiar importance of the unsaturated fatty acids in fat metabolism rests in the fact that they are apparently not only actively in process of metabolism, desaturation being the first stage in their metabolic change, but by their compounds they form an integral part of the protoplasm itself. Leathes believed that the purpose of desaturation was to render the acids more easily oxidized under bodily conditions as they undoubtedly are in the laboratory, that the double bonds provided points of weakness at which the long chains were broken, the fragments of which were then disposed of separately by β-oxidation. Dakin (13) has however given evidence to show that the unsaturated acids may not be more readily oxidized in the animal body than the saturated acids and it has been pointed out elsewhere (9) that there is no evidence in favor of the breaking of the chains at the double bonds. Nevertheless there is little doubt that desaturation does take place in the animal body and that it is to be regarded as the first stage in oxidation, a conception which is in agreement with the modern belief that oxidation is concerned with the activity of hydrogen quite as much as with the activity of oxygen. More recently Hopkins (14) has shown that unsaturated acids (linseed oil, cod liver oil) are oxidized under the influence of glutathione while the saturated acids are not. Hence information as to the nature and distribution of the fatty acids in the compounds found in various tissues is of first importance for an understanding of the behavior of the lipids in metabolism and the following communication represents a beginning in that direction. It contains data on the lipids of heart muscle, a tissue which is of especial importance in metabolism because of its continuous activity and high energy requirements. Other tissues of the same and different animals are in process of examination. The work is necessarily exploratory in nature and many problems are exposed, the solution of which must wait until their relative value is shown by the general survey.

EXPERIMENTAL.

The procedure for the examination of the tissue was as follows:

Preparation of Sample.—A whole beef heart, still warm from the slaughter-house, was freed from visible fat and non-muscle tissue and put twice through a meat grinder, the second time using the finest cutter.

Dehydration.—Of the finely ground material, 1 kilo (or the nearest hundred gm. 900, 800, etc.) was taken and well stirred into three volumes of warm 95 per cent alcohol and the mixture kept at about 35°C. for 2 hours, after which the solids were separated by filtration through a Buchner funnel.

Extraction.—After rinsing the dehydrated muscle on the funnel once with alcohol, it was divided into portions representing about 250 gm. of the original muscle and extracted with 95 per cent alcohol in a modification of the Clark hot extractor (15) using the quart size percolators. The extraction was divided into three periods of 1 hour each, at the end of each period the sample in the extractor being well stirred to loosen the material and the solvent changed. About 300 cc. of alcohol were used for each extractor and each period of extraction.

Recovery of the Lipids.—While the extraction was going on, the filtered alcohol used in dehydration was evaporated by distillation in a partial vacuum (about 20 mm. of mercury) until the alcohol was gone (as evidenced by the marked slowing in the distillation rate and the formation of droplets on the condenser tube). The distillation flask was kept at about 40°C. during the distillation by immersion in hot water. If the liquid in the flask was not allowed to rise above this temperature the foaming which is often a troublesome accompaniment of the distillation was entirely avoided. The milky liquid, amounting to about 250 cc. was transferred to a separatory funnel and the distillation flask rinsed clean with about 200 cc. of ether which was then poured into the separatory funnel and used to extract the liquid there; then after separation it was used to dissolve the lipids from the later extractions as below. The alcohol from the hot extractions was now transferred to the distillation flask and evaporated to dryness under the same conditions as above, after which the dry material was extracted with the ether used in the preliminary extraction. The flasks were rinsed out with

fresh ether and this ether used to complete the extraction of the watery liquid. The combined solvent was then transferred to a separatory funnel where it was left, generally overnight, in a cool place to allow water to separate out.

During the ether extractions the flasks, separatory funnel, etc., were flushed out with nitrogen or carbon dioxide gas and kept in dim light so as to diminish oxidation. The whole procedure up to this point was ordinarily carried out in 1 day and as noted below the extraction was quite good—only negligibly small amounts of lipid being left in the muscle.

Phospholipid Fraction.—The combined ether extracts (400 to 500 cc.[2]) were freed from the separated water (which was shaken out once with ether and the ether added to the main portion) and from a small portion of white material (sphingomyelin (?)—see below) and evaporated to 100 cc. The 100 cc. residue was transferred in approximately 25 cc. portions to four 100 cc. centrifuge tubes using a minimum amount of ether for rinsing, and the tubes filled to capacity with acetone, which was added in small portions with shaking. The tubes were then centrifuged to clearness, the precipitated phospholipids well kneaded with acetone, which served both to remove fatty material and for dehydration, then dissolved in dry ether. If the solution in ether was clear it was divided into four portions in centrifuge tubes and the precipitation and working out with acetone repeated. If the solution was not clear (which was generally the case) it was cleared by centrifuging, after which the separation of the phospholipids was carried through as above. The material insoluble in ether was added to the sphingomyelin from the first ether solution.

Separation of Lecithin from Cephalin. Analyses.—The phospholipid mixture after the second precipitation was dissolved in a minimum amount of dry ether, centrifuged to clearness, and the cephalin precipitated by the addition of excess of absolute alcohol in the same way as the phospholipids were precipitated by acetone. The alcoholic solution (lecithin) was evaporated to dryness in partial vacuum at 35–40°C., then dissolved in dry ether, and made up to a volume of 50 cc. The precipitate

[2] These and other figures are on the basis of 1000 gm. of muscle.

(cephalin) was freed from alcohol, then dissolved in dry ether, and made up to 50 cc.

Aliquot portions of 2 cc. each were pipetted off for weight and iodine number determinations (Hanus method) which were made immediately after evaporating the solvent.

The aliquot for weight was received in a weighed flask and the preliminary drying (about $\frac{3}{4}$ of an hour) carried out on a steam bath with a current of carbon dioxide. The drying was completed in a vacuum desiccator over calcium chloride to approximately constant weight. All samples gained weight slowly after reaching a minimum value, probably through oxidation. The minimum weight was taken as the actual weight although, since oxidation was undoubtedly continuous, this weight was probably slightly high.

Saponification.—The remaining portions of the lecithin and cephalin ($\frac{46}{50}$ of the whole) were transferred to a flask, the ether evaporated in a current of carbon dioxide, 100 cc. of water and 10 gm. of stick sodium hydroxide added, and the whole heated with a return condenser on the steam bath for 1 hour, after which an equal volume of alcohol was added and the saponification continued at the boiling temperature for 3 hours longer.

The cooled liquid was transferred to a separatory funnel and shaken out with half its volume of petroleum ether (boiling away completely below 55°C.) to extract the unsaponifiable matter. In these samples the unsaponifiable weighed only a few mg. and for that reason was not included in the data below.

The liquid was then acidified and completely extracted with petroleum ether. The combined petroleum ether extracts were adjusted to a volume of 200 cc. and suitable aliquots, 10 to 20 cc. depending on the fatty acid expected, taken for iodine number and weight, which were determined in the same way as for whole lecithin and cephalin above.

Separation of the Fatty Acids into Solid and Liquid Fractions.—The remaining portion of the fatty acid solution, $\frac{160}{200}$ to $\frac{180}{200}$ of the whole, was freed from petroleum ether, dissolved in alcohol, and the solid and liquid acids separated by the lead salt-alcohol method of Twitchell (16) in an atmosphere of nitrogen. The separated lead salts of the solid acids were purified by a single recrystallization from alcohol, after which the precipi-

tated material was suspended in water and the fatty acids separated by acidification with dilute nitric acid and extraction with ether. The ether extract was freed from acid by washing with water, then evaporated, dried, and weighed.

The combined alcoholic solutions after removal of the solid acids were evaporated in a partial vacuum and the residues treated with petroleum ether and dilute acetic acid in an atmosphere of nitrogen, for the separation of the liquid acids. The petroleum ether extracts were freed from lead and acid by washing first with dilute acetic acid and then with water and the extract made up to 200 cc. after which aliquot portions were taken (ordinarily 20 cc.) for weight and iodine absorption value. Throughout the separation of the liquid acids they were protected from oxidation by flushing out the separatory funnels, etc., with nitrogen gas.

Bromination of the Liquid Acids.—The remaining portion of the liquid acids was freed from solvent, dried in a stream of carbon dioxide, the residues dissolved in chilled dry ether, and bromine added to excess as shown by the reddish color of the solvent. The solution was kept cold during the bromine treatment by the application of ice. The mixture was allowed to stand in the ice chest overnight, then the precipitate was separated in weighed 15 cc. centrifuge tubes and after washing to whiteness with chilled ether, dried, and weighed, the weight representing hexa- and octobromo fatty acids. For the separation of the hexabromo from the octobromo compounds advantage was taken of the solubility of the hexabromo compound in benzene but since the amount obtained was always less than one-tenth of the precipitate and the solubility of the octobromo compound in benzene was unknown, this separation was not carried out in the later samples.

Acetone-Soluble Portion—Fat.—This material was divided into three or four fractions as follows: After the separation of the phospholipids the combined acetone extracts were adjusted to 350 cc. and set in the cold for 4 hours or overnight. A flocculent white precipitate separated which was collected by centrifugation, washed with small amounts of acetone until the washings were free from color, then dissolved in ether and analyzed as Fraction 1. The acetone solutions from the separation of Fraction 1 were concentrated to a volume of 150 cc. and again set in

the cold for the separation of Fraction 2. A further concentration to about 75 cc. gave Fraction 3. The mother liquor formed Fraction 4.

Fraction 1 when freed from solvent was always solid at room temperature and was fatty in appearance. Fractions 2 and 3 were liquid at room temperature and also fatty. Fraction 4 was generally soft and sticky.

Analyses.—The fractions were all treated as follows: The material after freeing from acetone was dried first on the steam bath in a current of inert gas and then to constant weight in a vacuum desiccator, after which it was weighed. Absolute alcohol was added, the substance dissolved by warming, and an excess of normal sodium ethylate added. The mixture was boiled for 4 hours with return condenser after which it was cooled and an equal volume of petroleum ether (boiling off completely below 55°C.) and an equal volume of water were added. After shaking and allowing the layers to separate, the petroleum ether was drawn off and the extraction repeated. The two extracts were united, washed with water, the solvent distilled away, and the residue dried and weighed, the weight giving the unsaponifiable matter. The watery alcoholic solution was next acidified with hydrochloric acid and the fatty acids extracted with petroleum ether in the same way as the unsaponifiable substance. The combined extracts were made up to a convenient volume and suitable aliquots taken for weight and iodine number. If the remaining portion of solvent contained less than 0.5 gm. of fatty acid nothing more was done with it. If it contained more than 0.5 gm. the solvent was evaporated and the mixed fatty acids separated into solid and liquid acids by means of their lead salts as above. The solid acids were separated and weighed, the liquid acids made to a convenient volume, and aliquots taken for weight and iodine number. The remaining portion of the liquid acids, if sufficient in amount, was dissolved in cold ether, brominated, and the bromine addition products separated as in the procedure for lecithin and cephalin.

Notes on the Procedure.

The aim throughout the work was to isolate and examine the compounds containing the unsaturated acids with as little chance

as possible for oxidation or other changes. Some sacrifice of accuracy to speed was considered justifiable in order to minimize these changes. Furthermore in view of the many technical difficulties in the way of an exact study of the situation it was felt that, at first, more could be gained by making a wide, if somewhat superficial, survey than by attempting to cover every phase of the subject at once. The problems which arose could be studied later in the order of their importance.

Extraction.—The actual extraction period was shortened to 3 hours during which the material was exposed to the vapor of the boiling alcohol. This short extraction gave very good results since practically all the lipid material was removed. In testing the completeness of the extraction the extracted material was collected, air-dried, and weighed (the residue from 1000 gm. of beef heart weighed 160 to 170 gm.). One-fourth of the total was weighed out into a beaker, 200 cc. of water and 40 gm. of stick sodium hydroxide added, and the mixture digested on the steam bath for 4 hours, after which unsaponifiable matter and fatty acids were determined. The weights of material from six extractions calculated to 1000 gm. of moist material were as follows:

Sample No.	Unsaponifiable.	Fatty acids.
1	0.052	0.032
2	0.060	0.600
3	0.240	0.066
4	0.016	0.020
5	0.044	0.548
6	0.028	0.012

The amounts of unrecovered lipid were variable, as would be expected, but never large enough seriously to affect the results.

Oxidation of the Fatty Acids during Separations.—In spite of the precautions taken to prevent it, there was undoubtedly some oxidation of the unsaturated acids during the separations. The possibility of oxidation during the early part of the treatment was slight. The tissue was obtained while still warm from the animal and the dehydration was brought about by warm alcohol in the short time of 2 hours. During the extraction the tissue was in an

atmosphere of alcohol vapor so that oxidation was probably not one of the changes which might have taken place. In the later stages of the separation, the isolation of the cephalin, lecithin, and other fractions, the saponification and the extraction of the fatty acids, some exposure to air and light, was however unavoidable and there was undoubtedly some oxidation at that time. The iodine values given are, therefore, to be regarded as minimum values. The following calculations support this conclusion.

Considering one of the samples (C below) on which more complete data are given, the iodine value of the intact cephalin was 102, the fatty acid content was found to be 62 per cent, and the iodine value of the fatty acids (assuming that the non-fatty acid residue of the cephalin had no iodine value) should have been $\frac{100}{64} \times 102 = 164$ while the value found was 137. The liquid acids constituted 77 per cent of the total acids and should have had (assuming that the solid acids and the residue had no iodine value) an iodine value of $\frac{100}{77} \times 137 = 177$ while the value found was 180.

Considering the lecithin in the same way, the iodine value of lecithin was 84, the calculated value for the fatty acids was 133, the value found was 107. For the liquid acids the value calculated was 187, the value found was 151.

Fractions.—It has been shown (17) that the acetone precipitation does not remove all the phosphorus and that some phospholipid passes over into the acetone-soluble portion. The fact that some substances containing a smaller percentage of fatty acid than fat were present in the supposedly phosphatide-free fractions was shown by the low percentage values for fatty acid in these fractions. The double precipitation of the phospholipids did free them from practically all the fatty material, cholesterol, etc., since the percentage of fatty acids in these compounds was close to the theoretical and the unsaponifiable material was negligibly small. The separation of cephalin from lecithin undoubtedly favored the latter owing to the appreciable solubility of cephalin in absolute alcohol, so that the values for lecithin should be somewhat smaller and those of cephalin somewhat larger than recorded.

The fractions of the phosphatide-free, acetone-soluble residue

varied greatly in amount in different samples but are of comparative value since the fractionations were conducted in a similar way in all. Quite definite substances or mixtures were obtained in the first two fractions, the first being always white and flocculent and, after separation, solid at room temperature; the second always a reddish brown oil which after removal of the solvent was liquid at room temperature. The third or fourth fraction—the mother liquor—when dried was generally a thick viscous liquid. As seen from the averages the main differences between the fractions consisted in the proportion of solid to liquid acid, the solid decreasing and the liquid increasing in the later fractions. The percentage of unrecoverable acid also decreased in the later fractions.

Separations of the Fatty Acids.—The lead salt-alcohol method leaves considerable to be desired when applied to the fatty acid mixtures from heart muscle. The method was designed to be used with mixtures in which the ordinary saturated and unsaturated acids of natural fats were the main ones concerned and as such has been found sufficiently accurate. The mixture of acids from tissues undoubtedly contains more complex acids, possibly partly oxygenated, which do not separate either as solid or liquid in the Twitchell process. The sum of the weights of solid and liquid acids never agreed with the weight of the original fatty acid mixture and the explanation for the deficiency was to be found in the dark colored sticky mass which forms when the lead solution was added to the alcoholic solution of the fatty acids and which was insoluble in boiling alcohol. Part of this residue was soluble in ether and the fatty acid content of this portion gave an iodine value of about 30. The material insoluble in alcohol generally contained 25 per cent or more of the total fatty acids and will undoubtedly repay more detailed examination. Eckstein (1) has reported a similar finding in human stored fat but the amount of unrecoverable fatty acid in his sample was relatively small.

Bromination.—The separation of the bromine derivatives was probably only approximate because the octobromo compound is not absolutely insoluble in ether. The percentages of these unsaturated acids are therefore minimum values.

Results.

Twenty-two samples in all were examined, of which for economy of space three analyses are given in detail and the results of the others as averages and variations. The three chosen represent three different levels of phospholipid content and are fairly representative of the whole series. The weights are in gm. per 1000 gm. of muscle.

Sample A, Apr. 1, 1925.

Phospholipid fractions.
- Cephalin, weight 7.270 gm.
  - Total fatty acids 67 per cent, iodine No. 137.
  - Liquid fatty acids 54 per cent of total fatty acids, iodine No. 179.
  - Solid fatty acids 9 per cent of total fatty acids.
  - Fatty acids forming ether-insoluble bromine addition products, 6 per cent of liquid acids.
- Lecithin, weight 7.560 gm.
  - Total fatty acids 62.2 per cent, iodine No. 109.
  - Liquid fatty acids 53 per cent of total fatty acids, iodine No. 139.
  - Solid fatty acids 17 per cent of total fatty acids.
  - Fatty acids forming ether-insoluble bromides—3.4 per cent of liquid acids.

Fat fractions.
- Fraction 1. Weight 1.040 gm.
  - Total fatty acids 90 per cent, iodine No. 37.
  - Liquid fatty acids 20 per cent of total fatty acids, iodine No. 42.
  - Solid fatty acids 26 per cent of total fatty acids.
  - Insoluble bromine fatty acids, trace.
  - Unsaponifiable, 0.036 gm.
- Fraction 2. Weight 4.580 gm.
  - Total fatty acids 90 per cent, iodine No. 71.
  - Liquid fatty acids 35 per cent of total fatty acids, iodine No. 103.
  - Solid fatty acids 27 per cent of total fatty acids.
  - Insoluble bromine fatty acids, trace.
  - Unsaponifiable, 0.210 gm.
- Fraction 3. Weight 5.740 gm.
  - Total fatty acids 74 per cent, iodine No. 82.
  - Liquid fatty acids 52 per cent of total fatty acids, iodine No. 100.
  - Solid fatty acids 14 per cent of total fatty acids.
  - Bromine-insoluble, none.
  - Unsaponifiable, 0.761 gm.
- Free fatty acids 0.270 gm., iodine No. 100.

Total fatty acids of phospholipid fractions, 9.670 gm., average iodine No. 124.

Total fatty acids of fat fractions, 9.238 gm., average iodine No. 73.
Liquid fatty acids of phospholipid fractions, 5.400 gm., average iodine No. 160.
Liquid fatty acids of fat fractions, 3.980 gm., average iodine No. 99.

Sample B, Sept. 21, 1925.

Phospholipid fractions.
Cephalin, weight 9.330 gm., iodine No. 90.
Total fatty acids 65 per cent, iodine No. 137.
Liquid fatty acids 70 per cent of total fatty acids, iodine No. 200.
Solid acids 10 per cent of total fatty acids.
Bromine-insoluble, 10 per cent of liquid acids.
Lecithin, weight 9.260 gm., iodine No. 83.
Total fatty acids, 71 per cent, iodine No. 100.
Liquid fatty acids 53 per cent of total fatty acids, iodine No. 147.
Solid fatty acids 9 per cent of total fatty acids.
Bromine-insoluble, 9 per cent of liquid acids.
Ether-insoluble residue (sphingomyelin (?)) 0.561 gm., total fatty acids 40 per cent.
Fat fractions.
Fraction 1. Weight 2.190 gm.
Total fatty acids 53 per cent, iodine No. 59.
Liquid fatty acids 17 per cent of total fatty acids, iodine No. 88.
Solid fatty acids 50 per cent of total fatty acids.
Unsaponifiable, 0.105 gm.
Fraction 2. Weight 2.266 gm.
Total fatty acids 90 per cent, iodine No. 78.
Unsaponifiable, 0.053 gm.
Fraction 3. Weight 4.130 gm.
Total fatty acids 70 per cent, iodine No. 45.
Liquid fatty acids 45 per cent of total fatty acids, iodine No. 96.
Solid fatty acids 52 per cent of total fatty acids.
Unsaponifiable, 0.140 gm.
Fraction 4. Weight 5.635 gm.
Total fatty acids 65 per cent, iodine No. 80.
Unsaponifiable, 0.731 gm.
Free fatty acids 0.132 gm., iodine No. 65.
Total fatty acids of phospholipid fractions, 12.600 gm., average iodine No. 124.
Total fatty acids of fat fractions, 9.764 gm., average iodine No. 66.

Sample C, Oct. 22, 1925.

Phospholipid fractions.
Cephalin, weight 8.00 gm., iodine No. 102.
Total fatty acids 62 per cent, iodine No. 137.

Liquid fatty acids 77 per cent of total fatty acids, iodine No. 180.
Bromine derivatives insoluble in ether, 9 per cent of liquid acids.
Solid fatty acids 16 per cent of total fatty acids.

Lecithin, weight 12.80 gm., iodine No. 84.
Total fatty acids 63 per cent, iodine No. 107.
Liquid fatty acids 57 per cent, iodine No. 151.
Bromine derivatives insoluble in ether, 7.8 per cent of liquid acids.
Solid fatty acids 21 per cent of total fatty acids.

Fat fractions.

Fraction 1. Weight 1.560 gm.
Total fatty acids 86 per cent, iodine No. 42.
Liquid fatty acids 21 per cent of total fatty acids, iodine No. 113.
Solid fatty acids 50 per cent of total fatty acids.
Unsaponifiable, 0.040 gm.

Fraction 2. Weight 6.53 gm.
Total fatty acids 94 per cent, iodine No. 80.
Liquid fatty acids 42 per cent, iodine No. 113.
Forming insoluble bromides 0.9 per cent of liquid acids.
Solid fatty acids 22 per cent of total fatty acids.
Unsaponifiable substance, 0.280 gm.

Fraction 3. Weight 3.125 gm.
Total fatty acids 80 per cent, iodine No. 92.
Liquid fatty acids 51 per cent, iodine No. 120.
Solid fatty acids 26 per cent of total fatty acids.
Unsaponifiable substance, 0.870 gm.

Total fatty acids of phospholipids, 13.024 gm., average iodine No. 120.
Total fatty acids of fat fractions, 9.152 " " " " 76.
Liquid fatty acids of phospholipids, 8.415 " " " " 164.
Liquid fatty acids of fat fractions, 4.000 " " " " 115.

Averages and Variations.

Analytical data from twenty-two samples (owing to missing data, these values represent ordinarily about eighteen samples). Gm. per 1000 gm. of moist tissue.

	Average.	High.	Low.
Phospholipid fractions.			
Cephalin:			
Weight	7.276	9.330	5.832
Iodine No.	101	110	90
Total fatty acids, per cent of weight	66	73	58
Iodine No.	124.5	150	80
Liquid fatty acids, per cent of total fatty acids.	61	80	46
Iodine No.	170	217	135
Solid fatty acids, per cent of total fatty acids.	14	20	10
Four-bond acids, per cent of liquid acids (ten samples)	6.4	11.0	2.8
Lecithin:			
Weight	9.128	12.800	6.500
Iodine No.	87	96	83
Total fatty acids, per cent of weight	64.6	71	58.5
Iodine No.	115.6	139	101
Liquid fatty acids, per cent of total fatty acids.	53	63	34
Iodine No.	148	170	126
Solid fatty acids, per cent of total fatty acids.	20	48	3
Four-bond acids (ten samples), per cent of liquid acids	5.64	10.0	2.8
Fat fractions:			
Fraction 1:			
Weight	1.800	4.369	0.440
Total fatty acids, per cent of weight	80.5	94	53
Iodine No.	52	74	27
Liquid fatty acids, per cent of total fatty acids.	31	52	21
Iodine No.	104	121	69
Solid fatty acids, per cent of total fatty acids.	42	56	23
Unsaponifiable substance, weight	0.078	0.148	0.034
Fraction 2:			
Weight	3.183	7.121	0.450
Total fatty acids, per cent of weight	85	100	57
Iodine No.	71	98	82
Liquid fatty acids, per cent of total fatty acids.	33	43	13
Iodine No.	106	130	83
Solid fatty acids, per cent of total fatty acids.	34	56	3
Unsaponifiable substance, weight	0.200	0.640	0.015

	Average.	High.	Low.
Fat fractions—Continued.			
Fraction 3 (ten samples):			
Weight	2.926	5.740	1.053
Total fatty acids, per cent of weight	76	92	51
Iodine No	85.4	129	45
Liquid fatty acids, per cent of total fatty acids.	39	65	52
Iodine No	98	126	70
Solid fatty acids, per cent of total fatty acids.	42	83	14
Unsaponifiable substance, weight	0.475	1.308	0.138
Fraction 4:			
Weight	2.558	4.482	0.265
Total fatty acids, per cent of weight	81	96	70
Iodine No	86	110	57
Liquid fatty acids, per cent of total fatty acids.	60	73	52
Iodine No	106	113	94
Solid fatty acids, per cent of total fatty acids.	19	26	11
Unsaponifiable substance, weight	0.422	0.720	0.234
Free fatty acids, weight	0.347	0.864	0.132
Iodine No	92	120	65
Total fatty acids, phospholipid fractions	10.7	13.25	8.0
" " " fat fractions	7.48	15.0	5.0

DISCUSSION.

Phospholipids.

Much has been said about the constancy of content of the tissues in their essential constituents and the prevailing opinion is that such a constancy of composition is real. Workers are, however, not always in agreement as to just what constituents are essential and therefore constant, especially as certain ones may be present both as actual working elements of the protoplasm and as more or less inert, stored material. With increasing knowledge of the fatty acid compounds and their occurrence in the living animal, there is a growing tendency to place them in the class of essential constituents and especially is this true of the phospholipids. At the same time the probable function of these compounds as intermediate stages in the metabolism of the fats cannot be ignored. In the samples examined, each one of which represented a single heart, the weights of cephalin varied between 5.83 and 9.33 gm. per kilo of tissue. The iodine value

of the cephalin itself varied between 90 and 110; of the total fatty acids of cephalin between 80 and 150; of the liquid acids between 135 and 217. Similar differences but not so marked were to be observed in the lecithin, although the weights of lecithin varied more widely than those of cephalin. These variations, up to nearly 100 per cent, can hardly be regarded as normal variations in the *composition* of a tissue and are believed to indicate rather that the phospholipids are in part at least, reserve material and as such would be expected to vary in different samples. Since fat is purely reserve material, it would be expected to be excessive in those samples having the greatest total lipid content but such was not found to be the case since the phospholipid fatty acids bear about the same relation to the fatty acids of the fat fractions whether the total amount is high or low.

The lecithin weights were always higher than the cephalin weights (average 9.128 gm. per 1000 gm. of muscle as compared with 7.276 gm. for cephalin), but taking into account the solubility of cephalin in absolute alcohol (or rather in the mixture of absolute alcohol, ether, and lecithin from which it was precipitated) the true values for the two are probably not far apart.

Certain other differences between the two may be noted. The fatty acid percentage is higher (66 compared with 64.6) for cephalin than for lecithin, as would be expected from the difference in molecular weight. The iodine values of the phospholipid itself as well as of the total fatty acids and the liquid fatty acids are higher in the cephalin fraction than in the lecithin fraction and the difference is most pronounced in the liquid fatty acids (170 for those from cephalin as compared with 148 for those from lecithin). The percentage of liquid acids is also higher in the cephalin fatty acids than in the lecithin acids (61 per cent as compared with 53 per cent) but the difference is proportionately not as great as the proportionate difference between the iodine values for the two, so that the cephalin fraction contains not only a higher percentage of unsaturated acid than the lecithin fraction but the acids are more unsaturated.

The solid (saturated) acids are present in much smaller proportion than the liquid acids—generally less than one-third—and are in smaller percentage in cephalin than in lecithin. The unrecoverable fatty acids (lead salts insoluble in boiling alcohol)

amount to about 25 per cent of the total in both lecithin and cephalin.

Allowing for loss in manipulation, an approximate division of the fatty acids in the phospholipids would be solid 15 per cent, liquid 60 per cent, unknown 25 per cent.

Acetone-Soluble Substances—Fat Fractions.

Fraction 1 began to separate from the acetone mother liquor soon after the phospholipids had been removed, as a white flocculent precipitate, and concentration of the acetone to about 350 cc. for each kilo of tissue resulted in its practically complete separation. When freed from solvent, it was solid at room temperature. Concentration of the acetone to half the above volume gave the second fraction as an oil which remained liquid when the solvent was evaporated away. The separation was not complete and it was a question whether the third and final fractions did not contain the same material. All the fractions yielded on saponification 80 to 85 per cent of their weight of fatty acid, the first and last fractions yielding lower amounts than the other two. If the fractions had consisted of fat alone the fatty acid content would have been about 10 per cent greater, so that they all contain a portion of some compound with a lower fatty acid content. The possibility of phospholipid passing over into these fractions has been mentioned. Since the phospholipids, such as lecithin and cephalin, yield about two-thirds of their weight of fatty acid, there would of necessity be a relatively large proportion of them present to lower the fatty acid values to the extent found. None of the known phospholipids would escape precipitation in such large amounts and it is, therefore, probable that the substances in question in these fractions represent other compounds. The presence of glycerides of the shorter chain fatty acids is probable and would help to explain the low fatty acid percentages found.

The iodine values of the mixed fatty acids of the fat fractions were all much below those of the fatty acids of the phospholipid fractions and, except for Fraction 1 which had a definitely lower value, were of about the same order (80 to 85) and were considerably higher than the values of the fatty acids of the stored fat in this animal. The liquid acids in all fat fractions had

nearly the same iodine value (100 to 105) which was again much below that of the liquid acids of the lecithin and cephalin. The similarity in iodine values of liquid acids for all the fat fractions indicated that about the same liquid acid mixture was present in all. The liquid acids all yield a slight amount (generally below 2 per cent of their weight) of ether-insoluble bromine derivatives, as compared with about 6 per cent for the phospholipids. The iodine values of the liquid acids indicated that while they consisted mainly of oleic acid, there was a noteworthy proportion of more unsaturated acids.

The fatty acid mixture of the first three fractions averaged about 40 per cent of solid acids, a value which is relatively constant for Fraction 1 but variable in the other two fractions. The percentage of unrecoverable fatty acid was somewhat lower in these than in the phospholipid fractions and occasionally it was possible to recover nearly all the fatty acids as either solid or liquid.

The sum of the fatty acids in combination in the phospholipids was of about the same order as the sum of those combined in the fat fractions although there was always more fatty acid in the phospholipids than in the fat. Thus in Sample A the fatty acids in the phospholipids were 9.67 gm. per kilo of moist tissue, in the fat 9.24 gm.; in Sample B, 12.60 and 9.76; in Sample C, 13.0 and 9.15; in the averages 10.63 and 8.61. The phospholipids contained a higher percentage of liquid (about double) and a lower percentage of solid (about half) fatty acids than the fat fractions.

As regards unsaturation, the fatty acids of the fat fractions stand midway between the fatty acids of the stored fat and those of the phospholipids, the percentage of unsaturated acids and the degree of unsaturation increasing in the following order —stored fat, tissue fat, lecithin, cephalin.

Unsaponifiable Substance.—In general the amount and percentage of unsaponifiable substance increased in the order of separation of the fractions, being least in the first fraction, increasing in the second and third, and about the same in the third and fourth. Much of the unsaponifiable matter was probably cholesterol and the results obtained (see below) indicate that it was present entirely in the free condition, a finding which is in

agreement with most of the data in the literature. Determinations of free and combined cholesterol were made on a few samples as follows:

An aliquot portion of the phospholipid-free acetone mother liquor was taken and divided into two equal parts. In one the acetone was evaporated, the residue taken up in alcohol, excess of alcoholic solution of digitonin added, and the mixture allowed to stand overnight. The precipitate was transferred to a weighed centrifuge tube, washed first with alcohol, then with ether, and dried and weighed. The other half of the aliquot was freed from solvent, dissolved in absolute alcohol, an excess of sodium ethylate added, and the solution boiled 4 hours with a return condenser. The unsaponifiable matter was separated, dissolved in a little alcohol and, in order that the conditions of precipitation might be the same, added to the residual liquid containing excess of digitonin from which the cholesterol of the first half of the aliquot had been precipitated. The mixture was allowed to stand overnight, then the digitonide was centrifuged off, washed, dried, and weighed as before. The weights (in gm.) divided by four were taken as the weights of cholesterol.

Results were obtained as follows:

No. 1.	Before saponification,	0.302	gm.	digitonide	=	0.076	gm.	cholesterol.
	After	"	0.261	"	"	= 0.065	"	"
" 2.	Before	"	0.238	"	"	= 0.060	"	"
	After	"	0.226	"	"	= 0.056	"	"
" 3.	Before	"	0.280	"	"	= 0.070	"	"
	After	"	0.260	"	"	= 0.065	"	"

In all cases the amount of material precipitable by digitonin was less after saponification than before, indicating the presence in the extracts of some substance with power of precipitating digitonin which was destroyed by saponification. Since cholesterol is known to be resistant to alkali, the material was probably not cholesterol. The values obtained gave no basis for believing that cholesterol esters were present.

Free Fatty Acids.—As in the work on the blood lipids, there was always obtained a small amount (averaging about 2 per cent of the total fatty acid of the sample) of fatty acid which could be washed out of the lipid mixture with dilute alkali and which therefore represented fatty acid which was either free in the muscle or

was split off during the separations. The iodine absorption value (average 92) was about the same as that of the fatty acids of the fat fractions and below that of the phospholipid fractions, indicating its relation to the fat rather than to the phospholipid. In blood plasma, the free fatty acid resembled more closely that of the phospholipid.

Bromine Derivatives of the Fatty Acids.—In the phospholipid fractions, the fatty acids yielding bromine derivatives insoluble in cold ether averaged about 6 per cent of the liquid acids or 3.5 per cent of the total acids. In the fat fractions they varied from unweighable amounts up to 6 per cent of the liquid acids, averaging 1.4 per cent. The ether-insoluble material consisted almost entirely of four-bond acids, as shown by the following experiment. The collected dried material from a number of precipitations was finely ground and extracted twice with cold benzene.

Weight of sample before extraction, 7.425 gm.
" " material soluble in benzene, 0.385 " 5.2 per cent.

The small percentage of material soluble in benzene indicated that the amount of three double bond acids was negligibly small and there was considerable doubt whether any at all was present, since the octobromide is not entirely insoluble in benzene.

In dog heart muscle Wesson (5) found 100 mg. of arachidonic acid per 1000 gm. of tissue and while the results of the present work furnished no data on the whole muscle, the fatty acids of the phospholipid fraction yielded about 6 per cent of insoluble bromo acids. On this basis 1000 gm. of tissue would yield about 350 mg. of four-bond acid in the phospholipid fraction.

The exact nature of this four-bond acid is unknown, but judging by the work of Hartley and Wesson it is probably arachidonic acid. This is a C_{20} acid and brings to mind the recent work of Armstrong and Hilditch (18) who found that the four-bond acids of whale oil were always C_{20} or higher. It was shown by these authors that the 9 to 10 position of the carbon atom from the carboxyl is the place of choice for the first double bond in a fatty acid chain, which was also noted by André (19). The latter author also brought out the interesting fact that in the naturally occurring unsaturated acids, the succeeding double

bonds after the first all come on the side of this first double bond away from the carboxyl and in a position not closer than the second carbon to each other. If these findings are true at least a C_{20} chain would be necessary in order to get four double bonds into the molecule.

Thus, starting with ordinary oleic acid, the degrees of unsaturation would be represented as follows:

$CH_3 \cdot CH_2 \cdot CH_2 \cdot CH_2 \cdot CH_2 \cdot CH_2 \cdot CH_2 \cdot CH_2 \cdot CH = CH \cdot (CH_2)_7 \cdot COOH$, 1-bond.
$CH_3 \cdot CH_2 \cdot CH_2 \cdot CH_2 \cdot CH_2 \cdot CH = CH \cdot CH_2 \cdot CH = CH \cdot (CH_2)_7 \cdot COOH$, 2- "
$CH_3 \cdot CH_2 \cdot CH = CH \cdot CH_2 \cdot CH = CH \cdot CH_2 \cdot CH = CH \cdot (CH_2)_7 \cdot COOH$, 3- "
$CH_2 \cdot HC = CH \cdot CH_2 \cdot CH = CH \cdot CH_2 \cdot CH = CH \cdot CH_2 \cdot CH = CH \cdot (CH_2)_7 \cdot COOH$, 4-bond.

Nature of the Unsaturated Acids.

Any attempt to give an exact quantitative picture of the unsaturated acids from the data on the bromo derivatives and from the iodine absorption values encounters the difficulty that the behavior of the tissue acids with bromine is not exactly known and the extent of oxidation during the manipulations is also an unknown factor. Acids with three double bonds may, however, be eliminated from consideration because they were found in such small amounts (about 5 per cent of the weight of the four-bond acids) that there was a question of their presence at all. These findings are in agreement with those of Ellis and Hankins (2) who reported no triple bond acids in the meat fat of hogs and with those of Eckstein (1) who found that they constituted not more than a possible 10 per cent of the weight of the four-bond acids in human depot fat. Of the other unsaturated acids, those with four double bonds may presumably be determined directly from the weight of the ether-insoluble bromo derivative, although there may be four-bond acids of which the bromine derivatives behave differently.

The amounts of acids with two double bonds and of those with single bond may be calculated from the iodine values of the liquid fatty acid mixture after correcting for the four-bond acid present. The values obtained are undoubtedly low for all the unsaturated acids except oleic acid because of oxidation. The calculated percentages for linolic acid (two double bond acid) made from the iodine values of mixed liquid acids are:

	Cephalin.	Lecithin.
	per cent	*per cent*
Sample A	80	44
" B	93	38
" C	74	46
From the averages	70	48

As may be seen they are of about the same order for each of the two phospholipids and illustrate what is probably the main difference between them—a difference in the content of linolic acid. As regards the liquid acids in the fat fractions they appear to be nearly the same in composition for all fractions, having an average iodine value of not far from 100. Neglecting the small and variable content of four-bond acids the linolic acid content of these would be about 15 per cent.

SUMMARY.

The largest proportion of unsaturated acids in beef heart muscle was found in the cephalin fraction, the next largest in the lecithin fraction, and the lowest in the remaining fractions which are mainly fat.

The total unsaturation was in the same order, the iodine value of the liquid acids averaging 170 for cephalin, 148 for lecithin, and about 100 for the fat fractions.

Without taking into account the oxidation during manipulation, the extent of which cannot at present be estimated but which was probably considerable, the distribution of the various types of fatty acid in the fractions was found to be about as follows: The phospholipid fatty acids consisted of 10 to 20 per cent of saturated, 50 to 70 per cent of unsaturated, and about 25 per cent of acids of unknown nature. In the fat fractions, the unsaturated acids averaged about 41 per cent, the saturated acids 34 per cent, and the unknown acids about the same as in the phospholipids.

Of the unsaturated acids, the important ones appeared to be oleic, linolic, and arachidonic acids and the proportionate distribution in the fractions was approximately as follows: Cephalin contained arachidonic 6 per cent, and the rest mainly linolic; lecithin, a little less arachidonic, 45 per cent linolic, and the

rest oleic; fat, less than 2 per cent arachidonic, about 15 per cent linolic, and the remainder oleic acid. Acids with three double bonds were absent. The values for linolic and arachidonic were undoubtedly minimal because of oxidation during manipulation.

Cholesterol esters appeared to be absent from heart muscle which is in marked contrast to beef plasma where they were the most important carriers of the unsaturated acids.

Free fatty acids were present in varying amounts, averaging about 2 per cent of the total acids and with an iodine value of about the same order as the fat fractions.

The data show that the lipid constituents of heart muscle varied widely in amount, in the nature of their contained fatty acids, and in relation to each other. In general the weight of lecithin was greater than that of cephalin and the weight of fatty acid in the phospholipid fractions was greater than that in the fat fractions. Both lecithin and cephalin varied considerably as to the proportion of solid to liquid acid in their molecules, but the unsaturated component of each tended to have the same composition. In about half the samples, the sum of the fatty acids in the total phospholipid was constant between 9 and 10 gm. per 1000 gm. of muscle.

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INVESTIGATIONS IN TETANY.*

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Research on experimental and clinical tetany in recent years has to a large extent been dominated by the view that tetany occurs when the calcium ion concentration falls below a certain threshold on account of a change in the reaction of the blood towards the alkaline side. In support of this theory use has been made, amongst other things, of the equation given by Rona and Takahashi (18)

$$\frac{[Ca^{++}] \cdot [HCO_3^-]}{[H^+]} = K$$

which is valid for saturated watery solutions of calcium bicarbonate.

As will be seen from the equation a shift of the reaction in an alkaline direction causes a fall in the calcium ion concentration, and a shift in an acid direction, a rise.

This alkalosis theory has had further support from the fact that acid treatment, as for example the administration of $CaCl_2$ and NH_4Cl, in addition to making the blood temporarily more acid, also causes the total calcium in the serum to rise and the clinical symptoms to disappear.

Quite recently an extensive investigation by Turpin (24) in France has appeared, which on the whole gives clinical support to these more hypothetical theories, since he finds a more alkaline pH and a greater alkali reserve in tetany than in normal persons.

* The determinations of the pH and $BHCO_3$ and the calculations of the $[Ca^{++}]$ were carried out by Paul Drucker. The calcium and phosphorus analyses were done by Frans Faber.

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Turpin, however, compares the values found by himself in tetany with normal values obtained elsewhere for adults without, as far as one can see, having himself determined the normal values, at any rate of the pH, with his own technique and for children. Moreover, in his material of 50 cases there are only four where determinations were made on the same child during tetany and after its disappearance. On these grounds it does not seem to us that these experiments can rightly be taken as confirmation of the alkalosis theory, especially as only two of the cases show more marked alkaline values during the disease than after it.

In order to throw more light on this theory we have determined simultaneously the actual pH of the blood, its alkali reserve, total calcium, and acid-soluble P and the calcium ion concentration calculated with the Rona-Takahashi equation in twenty small children with tetany, while the disease was manifest and also after it was cured. Further, we have made determinations during the course of the disease, during $CaCl_2$, NH_4Cl, and calcium lactate treatment, during bicarbonate and phosphate administration, and lastly during and after light treatment.

Technique.

The pH determinations were made by the method described by Cullen (2) with Hawkins' (10) modification using the technique recently described by Drucker and Cullen (3). The determinations of the alkali reserve were done by Van Slyke's (25) titration method, the total calcium determinations by Tisdall's (23) modification of Kramer and Tisdall's method, and the P determinations by Tisdall's (22) method. The blood samples were all taken by heel puncture after about 8 hours fasting. The diet consisted of milk foods and oatmeal gruel five times and twice daily respectively. All the determinations were duplicated apart from the P determinations and some of the bicarbonate determinations.

I. Reaction of Blood in Tetany and Relation between This, Total Calcium, and $[Ca^{++}]$.

The first problem we set ourselves was: Do we find so great a displacement of the reaction of the blood in the alkaline direction in manifest tetany, that in virtue of the decreased calcium ion

concentration we can explain the occurrence of tetany? Our results lend no support to this because we find no signs of alkalosis when compared with a number of normal children or when compared with the reaction of the blood in the same child after it was cured.

TABLE I.

pH			$BHCO_3$		$[Ca^{++}]$ (calculated).	
Tetany.	After tetany.		Tetany.	After tetany.	Tetany.	After tetany.
			vol. per cent	*vol. per cent*	*mg. per cent*	*mg. per cent*
7.31	7.29	−0.02	39.9	46.4	3.9	3.5
7.42	7.37	−0.05	47.9	48.8	2.5	2.7
7.45	7.39	−0.06	41.0	50.2	2.7	2.5
7.39	7.39	0	37.2	45.7	3.4	2.8
7.37	7.39	+0.02	39.9	40.3	3.4	3.2
7.46	7.43	−0.03	38.5	45.3	2.8	2.6
7.37	7.43	+0.06	36.5	56.2	3.7	2.1
7.43	7.42	−0.01	45.3	42.4	2.6	2.8
7.36	7.40	+0.04	42.2	45.3	3.3	2.8
7.50	7.45	−0.05	52.9	44.2	1.9	2.5
7.38	7.37	−0.01	46.3	47.1	2.8	2.8
7.40	7.40	0	48.2	43.9	2.6	2.8
7.30	7.42	+0.12	36.9	41.4	4.3	2.9
7.40	7.47	+0.07	36.3	41.4	3.4	2.6
7.40	7.41	+0.01	43.9	49.5	2.8	2.5
7.39	7.42	+0.03	32.9	36.9	3.9	3.2
7.47	7.41	−0.06	42.6	48.9	2.5	2.5
7.38	7.41	+0.03	38.1	54.0	3.4	2.3
7.41	7.32	−0.09	43.1	47.5	2.8	3.2
7.35	7.39	+0.04	41.0	42.3	3.4	3.0
Average. 7.40	7.40	+0.002	41.5	45.9	3.1	2.8
(7.41)*			(46.9)*		(2.6)*	

* Average from thirty-five normal children.

As will be seen from Table I, the pH values are the same in manifest tetany and under normal conditions. The same practically applies to the alkali reserve of the blood; in fact, this is slightly less in tetany than after the disease is cured.

If we observe what happens to the calcium ion concentration calculated by the Rona-Takahashi equation during and after

tetany, we find that the calcium ion concentration is rather greater during manifest tetany than after it is cured, which was only to be expected from the values found for the alkali reserve. This

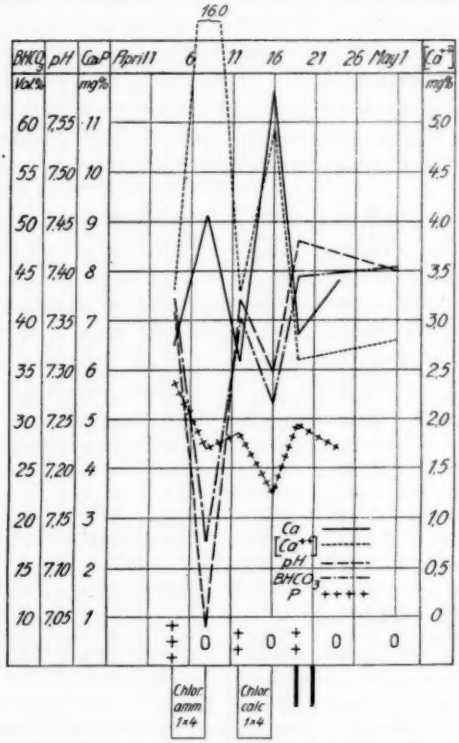

CHART I. Chart I shows the action of ammonium chloride and calcium chloride. It will be seen that ammonium chloride affects the reaction of the blood—both the actual (pH 7.04) and the potential—to a considerably greater extent than calcium chloride, whereas calcium chloride produces a far greater rise in the total calcium with a corresponding fall in the acid-soluble phosphorus. The high values of the calculated calcium ion concentrations are also noteworthy, especially after the ammonium chloride treatment (16.0) as compared with the value found for the total calcium (9.1). Lastly it will be observed how the light treatment makes all the values tend towards the normal value.

difference is, however, so slight that even on the assumption that the Rona-Takahashi equation applies to tetany (which we shall return to presently) no real significance can be attributed to it.

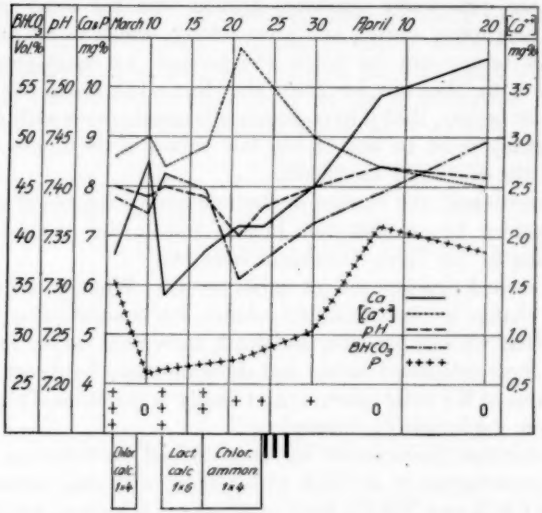

CHART II. Chart II, like Charts I and V, shows that calcium chloride is capable of raising the total calcium to a considerably greater extent than ammonium chloride in spite of the latter's stronger acidosis effect. Further, the action of calcium lactate will be seen, and the favorable and permanent action of light treatment during which the total calcium rises to normal while the clinical symptoms disappear, and finally the sharp rise in the total calcium after the administration of calcium chloride will be observed, *in spite of the unaltered blood reaction*, which is also the case after the administration of calcium lactate, although to a lesser degree.

Explanation of Symbols in Charts.

Chlor. calc., dried calcium chloride.
Chlor. amm., ammonium chloride.
Lact. calc., calcium lactate.
P.F., primary sodium phosphate NaH_2PO_4.
S.F., secondary sodium phosphate Na_2HPO_4.
Bicarb. natr., sodium bicarbonate.
I denotes general radiation with quartz lamp.
1 × 4 denotes 1 gm. four times daily.
\+ denotes the severity of the tetany.
0 denotes no clinically demonstrable tetany.

The value 2.8 mg. per cent moreover agrees well with the normal values found by other authors, as for example Bigwood (1).

If we now compare these calculated calcium ion concentrations which are, practically speaking, normal with the total calcium values also found during and after tetany, which in all cases of manifest tetany are far below the normal, we must conclude, always with the proviso that the Rona-Takahashi equation applies to tetany, that if hypocalcemia is synonymous with tetany then tetany must be due to too low values of the undissociated part of the calcium in the blood.

As mentioned, the change in the reaction of the blood will be accompanied by an alteration in the calcium ion concentration according to the Rona-Takahashi equation.

The second problem we set ourselves was: What is the extent of the change in the calculated calcium ion concentration of the blood that we can produce, and what agreement do we find between these calculated values and those obtained by direct determinations of the total calcium; and finally is the Rona-Takahashi equation applicable to tetany?

To elucidate these points we have devised the following provisional experiments in children with tetany, with oral administration of $CaCl_2$ and NH_4Cl, both of which, as is known, are able to change the reaction of the blood. The experiments extended over 2 to 4 days with the administration of 1.0 gm. four times daily in a 10 per cent solution, the last dose being given 1 hour before the blood sample was taken.

With regard to the calculated calcium ion concentration (Charts I and V), we find high values during the acid treatment, in fact in a few cases values (16.0 mg. per cent) which even considerably exceed the total amount of calcium found by direct determination, a result which shows that the blood under these conditions cannot be saturated with calcium, as the total quantity in such a case would be at least equal to or, more correctly, greater than the concentration of the ionized calcium.

The Rona-Takahashi equation which, as stated, only applies to saturated solutions, *cannot, therefore, be used to determine the ionized calcium in tetany when the blood is rendered acid.*

That it cannot be employed in tetany with a normal blood reaction either, follows from later experiments which show that

the blood again is not saturated with calcium under these conditions.

If we next look at the curves of the pH and alkali reserve values (Charts I, II, and V) the acidosis effect of $CaCl_2$ and NH_4Cl (7.04) will be clearly seen. Further, it will be observed how the total calcium rises with an acid reaction and falls again when the reaction becomes more alkaline. But there is another fact of interest which reappears in all our experiments; namely, that the

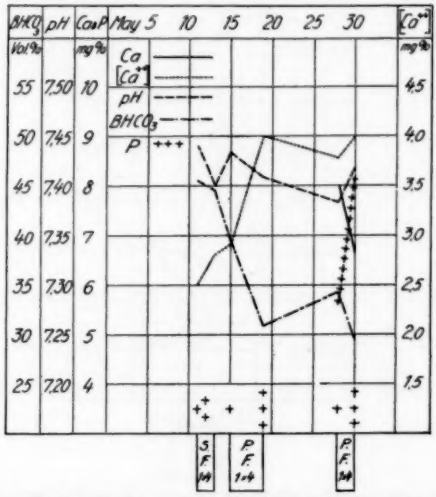

Chart III. Chart III shows the tetanigenous action of both NaH_2PO_4 and Na_2HPO_4, even with a change in the reaction of the blood to the acid side. No regular agreement between the kind of phosphate and the reaction of the blood is observed. The last experiment shows the antagonism between the total calcium and the acid-soluble phosphorus.

total calcium always rises higher with the $CaCl_2$ administration than with the NH_4Cl, in spite of the reaction being the same or even more acid after the dose of NH_4Cl.

We think that this justifies the same conclusion that we arrived at previously by another method, that *the blood is not saturated with calcium when it is made acid in tetany*, as it would otherwise take up equal amounts of calcium for the same shift in the reaction and with more acid reactions even larger amounts.

That the blood *cannot be saturated with calcium in tetany at the normal reaction of the blood either* is shown by two experiments (see Chart II) with the administration of $CaCl_2$ and calcium lactate, where a considerable rise in the total calcium occurs in spite of no change in the reaction of the blood.

The higher values for the total calcium with the administration of $CaCl_2$ further show that the therapeutic action of $CaCl_2$,

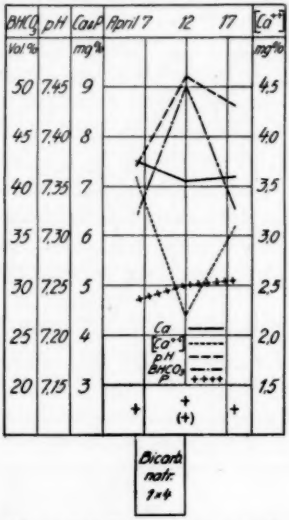

CHART IV. Chart IV exhibits the action of sodium bicarbonate. Although the reaction of the blood is shifted distinctly in the alkaline direction only a trifling clinical aggravation of the tetany, corresponding to a slight fall in the total calcium, is observed in spite of a large fall in the calculated $[Ca^{++}]$.

as was to be expected, is to be regarded as a combination of an acid and calcium effect and one which we do not find in the more weakly acting calcium lactate.

In accordance with this calcium chloride must, therefore, be regarded as the most rational symptomatic remedy in tetany.

We have tried the oral administration of sodium bicarbonate in only two cases of latent tetany, and found a displacement of the reaction of the blood in an alkaline direction attended by a slight

fall in the total calcium, but a rise in the amount of P. Only in one case did we observe an increase in the electrical hypersensitiveness, but otherwise no aggravation of the clinical condition in spite of a marked fall in the $[Ca^{++}]$ (see Chart IV) (compare Tezner (20)).

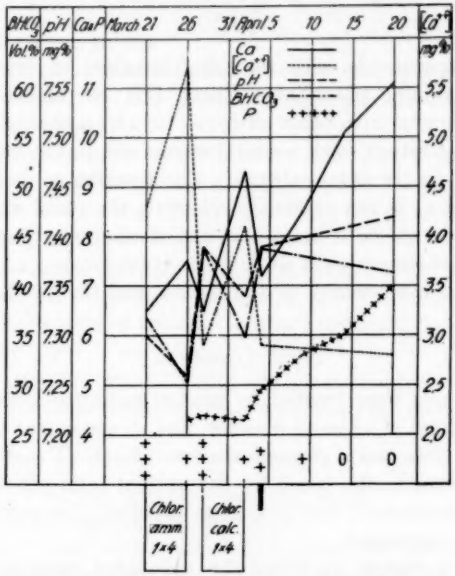

CHART V. Chart V, like Charts I and II, shows the action of calcium chloride and ammonium chloride. *In addition the striking effect of a single light bath lasting 15 minutes is seen.*

II. Relation between Total Calcium and Acid-Soluble Phosphorus during Acid Treatment.

If we consider the relation between the total calcium and the acid-soluble P during the acid treatment (Chart I) we observe, as earlier authors (11) have done, that a rise in the amount of calcium is accompanied by a fall in the amount of P and *vice versa*, which may be assumed to depend upon the fact that the organism in its efforts to preserve its reaction reacts upon the acidosis produced by these substances partly by excreting large

quantities of P in the urine chiefly in the form of primary phosphates.

In the case of the $CaCl_2$ treatment there is probably the additional factor that insoluble $Ca_3(PO_4)_2$ is formed in the intestine.

III. Experiments with the Administration of NaH_2PO_4 and Na_2HPO_4.

In the experiments with the administration of primary and secondary sodium phosphate (Chart III) by mouth we find, also in agreement with other authors (12, 14), a clinical aggravation of latent tetany with a simultaneous rise in the acid-soluble P and a fall in the total calcium. The reaction of the blood on the other hand is not markedly affected; the small changes we observed occurred both in an acid and alkaline direction, so that again in these experiments we have been unable to find any relation between the severity of the tetany and the reaction of the blood.

Light Treatment.

All our cases were treated by general radiation with a quartz lamp for a total of 15 to 45 minutes, and in agreement with other authors we obtained a *permanent* cure (Charts II and V) which was manifested in the blood by the normal total calcium value, while the reaction of the blood, as previously mentioned, was practically unchanged.

As already stated, we found the calculated calcium ion concentration after the cure of the tetany by light treatment about the same as the normal values given by other authors, which in conjunction with the normal values of the total calcium (values which, as is known, are only appreciably exceeded temporarily) might indicate that the blood must now be nearly saturated with calcium.

The antagonistic relation between P and Ca with the acid and phosphate treatment does not occur with the light treatment, since with the latter the low P value also rises.

RÉSUMÉ.

1. Tetania infantilis cannot be explained by an "alkalosis" of the blood since neither are the pH values more alkaline nor the

alkali reserve greater when the tetany is manifest than when it is cured. The theory that hypocalcemia, and hence tetany, is due to a change in the blood reaction in an alkaline direction cannot, therefore, be substantiated.

2. The Rona-Takahashi equation, which was evolved for saturated aqueous solutions *in vitro*, cannot be applied to blood plasma *in vivo* in tetany.

3. The blood in tetania infantilis is not saturated with calcium.

4. Treatment with $CaCl_2$ must be regarded as a combined acid and calcium therapy.

5. Investigations by other workers into the effect of the administration of acid and phosphates on the reciprocal relation between the calcium and phosphates of the blood, as well as on the clinical symptoms, are confirmed.

6. In two cases of latent tetany the disease could not be made manifest by the oral administration of moderate doses of sodium bicarbonate in spite of the shift in the reaction of the blood in an alkaline direction.

7. After general radiation with the quartz lamp the blood must be considered to be practically saturated with calcium.

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A QUANTITATIVE STUDY OF THE STORAGE OF VITAMIN A.*

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(Received for publication, February 16, 1926.)

The possibility that vitamin A could be stored in the animal body was suggested in 1913 by Osborne and Mendel;[1] and in 1915 McCollum and Davis[2] remarked upon differences in lengths of time that different animals of the same species (rats) could continue to grow upon diets lacking this factor. In 1920 Drummond and Coward[3] emphasized the importance of depleting the bodily store of this substance in experimental animals which are to be used in testing for it. Sherman, MacLeod, and Kramer[4] have pointed out the fact, which has been developed further by Sherman and Kramer,[5] that animals kept under like conditions, upon the same vitamin A-free diet, differ in survival periods according to the extent of the opportunity which they had enjoyed to store vitamin A from their previous diets. Steenbock, Sell, and Nelson[6] show a difference in the rate of growth of rats on a diet deficient in vitamin A depending upon the richness of the previous diet in this vitamin. Goldblatt and Soames,[7] also Sherman and Boynton,[8] show variations in the vitamin A contents of rat livers as determined by feeding weighed portions of these as sole source of the vitamin to test animals.

That age has an important influence in determining the extent of the

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[1] Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1913, xv, 311.

[2] McCollum, E. V., and Davis, M., *J. Biol. Chem.*, 1915, xx, 641.

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[7] Goldblatt, H., and Soames, K. M., *Biochem. J.*, 1923, xvii, 446.

[8] Sherman, H. C., and Boynton, L. C., *J. Am. Chem. Soc.*, 1925, xlvii, 1646.

storage of vitamin A in the body has been shown by Sherman and Storms[9] who placed rats from the same stock and the same previous diet, upon the same vitamin A-free food at different ages and found the survival period to increase with the age of the rat up to 6 months, the age at which the body has approximately reached its adult size.

The experiments to be described in the present paper were designed to study further the extent to which vitamin A can be stored in the body, and the times and conditions of feeding necessary to induce maximum storage in experimental animals of different ages.

EXPERIMENTAL.

In all of the present experiments, the extent of the storage of vitamin A in the body of the experimental animal was judged by determining the length of time it could survive without vitamin A when given a diet fully adequate in other respects and kept in individual cages with raised screen floors to prevent access to excreta. The vitamin A-free diet used was a mixture of: extracted casein, 18 per cent; dried yeast, 5; starch, 72; Osborne and Mendel[10] salt mixture, 4; sodium chloride, 1 per cent. This is the same as Diet 379 used by Sherman and Munsell[11] as the basal vitamin A-free diet for quantitative determinations of vitamin A in foods. The extraction of vitamin A from this diet probably removes the antirachitic vitamin also, but this evidently did not complicate the present studies of vitamin A for parallel experiments with and without ultra-violet irradiation were found to yield the same results. Undoubtedly all the animals contained considerable stores of antirachitic vitamin in their bodies when placed on the vitamin A-free diet. That lack of vitamin A was the cause of death in these experiments was confirmed by careful daily observation of the experimental animals and further examination at autopsy for such signs of deficiency of vitamin A as the characteristic abscesses in the glands at the base of the tongue and the frequency of inflammatory infections of the lungs, the sinuses,

[9] Sherman, H. C., and Storms, L. B., *J. Am. Chem. Soc.*, 1925, xlvii, 1653.

[10] Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1919, xxxvii, 572.

[11] Sherman, H. C., and Munsell, H. E., *J. Am. Chem. Soc.*, 1925, xlvii, 1639.

and the middle ear. The autopsy findings taken in conjunction with the premortem observations upon the eyes, the respiration, the digestion, and the condition of the skin and hair left no doubt that the time of death was determined essentially by the exhaustion of the bodily store of vitamin A.

All of the experimental animals were albino rats of the same stock and of accurately known age, pedigree, and nutritional history. For all of the experiments described in this paper the rats were taken from families which had been fed our Diet B (Laboratory No. 13) which is a mixture of one-third whole milk powder and two-thirds ground whole wheat with table salt in the proportion of 2 per cent of the weight of the wheat, the whole so ground and mixed as to ensure the consumption of the constituents

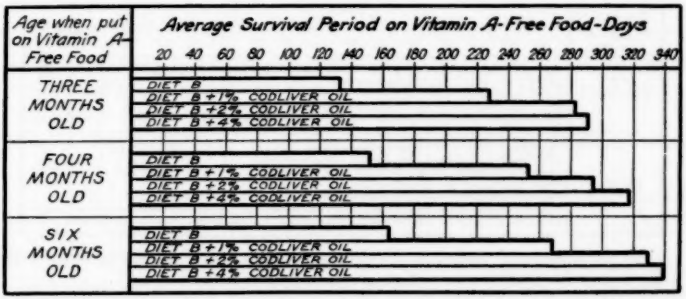
Age when put on Vitamin A-Free Food
Average Survival Period on Vitamin A-Free Food-Days
20 40 60 80 100 120 140 160 180 200 220 240 260 280 300 320 340
THREE MONTHS OLD
DIET B
DIET B +1% CODLIVER OIL
DIET B +2% CODLIVER OIL
DIET B +4% CODLIVER OIL
FOUR MONTHS OLD
DIET B
DIET B +1% CODLIVER OIL
DIET B +2% CODLIVER OIL
DIET B +4% CODLIVER OIL
SIX MONTHS OLD
DIET B
DIET B +1% CODLIVER OIL
DIET B +2% CODLIVER OIL
DIET B +4% CODLIVER OIL

FIG. 1. Effects of moderate and of increasingly intensive feeding of vitamin A upon the storage of this vitamin in the body at different ages.

of the diet in the exact proportions stated. The food mixture and distilled water were furnished *ad libitum*. The food consumption at different ages of the animals of our colony on this diet has been given in a previous paper.[12]

In the first series of experiments here reported, rats which had been separated from their mothers at the age of 28 days were continued either upon Diet B alone, or upon this diet plus 1, 2, or 4 per cent of cod liver oil and then at the age of 3, 4, or 6 months transferred to the vitamin A-free diet above described and kept under identical conditions for the determination of their survival periods. The results are summarized in Fig. 1. Taking the survival pe-

[12] Sherman, H. C., and Campbell, H. L., *J. Biol. Chem.*, 1924, lx, 5.

riods as measures of the relative amounts of vitamin A which had been stored under these different conditions of age and feeding, it is clear that at each age studied the animals which had received the richest intake of vitamin A had acquired the largest bodily store, and that at any given level of feeding, the bodily store became larger at 4 months of age than at 3, and larger at 6 months than at 4.

It is not to be inferred, however, that the body stores quantitatively the entire surplus received in the intake, for the increases in survival period found are, while very striking and doubtless extremely important, not arithmetically proportional to the concentration of vitamin A in the food.

Evidently the limit of the body's capacity for storage of vitamin

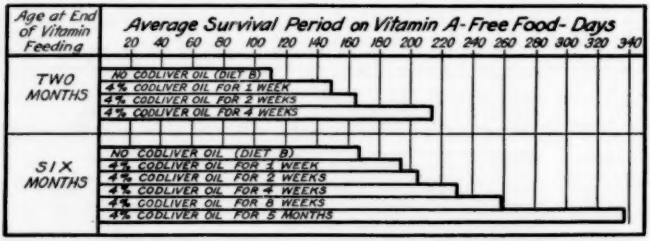
Age at End of Vitamin Feeding
Average Survival Period on Vitamin A-Free Food- Days
20 40 60 80 100 120 140 160 180 200 220 240 260 280 300 320 340
TWO MONTHS
NO CODLIVER OIL (DIET B)
4% CODLIVER OIL FOR 1 WEEK
4% CODLIVER OIL FOR 2 WEEKS
4% CODLIVER OIL FOR 4 WEEKS
SIX MONTHS
NO CODLIVER OIL (DIET B)
4% CODLIVER OIL FOR 1 WEEK
4% CODLIVER OIL FOR 2 WEEKS
4% CODLIVER OIL FOR 4 WEEKS
4% CODLIVER OIL FOR 8 WEEKS
4% CODLIVER OIL FOR 8 MONTHS

FIG. 2. Effects of intensive feeding of vitamin A for different lengths of time upon the bodily store of this vitamin at different ages.

A is not quickly reached, whether regarded from the standpoint of the level of intake or the length of time during which a liberal intake is continued; and it is possible, by building up a bodily store through previous feeding, to prolong very greatly the length of time that an animal can survive upon a diet devoid of vitamin A.

In a second series of experiments the question of the rapidity with which the body can accumulate a large store of the vitamin was studied in another way. Rats receiving Diet B were given in addition to this diet 4 per cent of its weight of cod liver oil for different lengths of time before reaching a standard age of 2 or of 6 months. Thus rats were placed upon the vitamin A-free diet at 2 months of age, some of which had received Diet B only, while others had received an addition of 4 per cent of cod liver oil for the preceding 1, 2, or 4 weeks. Similarly the 6 months old rats had

received Diet B only, or with the cod liver oil addition for the 1, 2, 4, or 8 weeks or for the 5 months immediately preceding the attainment of the standard age of 6 months at which they were all placed upon the vitamin A-free diet. The results are summarized in Fig. 2.

Here again it is found, by experiments of this somewhat different kind, that the body, whether tested during the time of its most rapid growth or when growth is nearly complete and has become relatively much less rapid, does not quickly acquire the full maximum store of vitamin A of which it is capable. Maximum storage in the literal quantitative sense evidently requires considerable time (especially when considered in the light of the rapidity with which the rat passes through his life cycle) even when the level of intake of the vitamin A is high.

Nevertheless, storage under such conditions is rapid in the sense that in a short time the body was able to increase its store very considerably as shown by the subsequent survival periods upon vitamin A-free food.

In a supplementary experiment a litter of 28 day old rats was divided, one being put upon the vitamin A-free food at once and three others after receiving food containing 4 per cent of cod liver oil for 2, 4, and 7 days, respectively. The 2 days feeding resulted in prolonging the survival period by 30 days or over 50 per cent; the 4 days feeding prolonged it about 90 per cent; the 7 days feeding, about 120 per cent.

Another litter was depleted of its surplus vitamin A by keeping on the vitamin A-free diet until growth ceased; then two of these rats were left upon the same diet while two were given access to clear cod liver oil for 1 day and then returned to the vitamin A-free diet. The 1 day's feeding of cod liver oil increased the average survival period by more than 50 per cent over that of the controls. It was not feasible in this experiment to ascertain the actual weight of cod liver oil consumed.

These latter experiments, although only preliminary, are of interest not only in their bearing upon the problem of the rapidity with which the body can store vitamin A but also as suggesting the possibility of quantitative studies of the vitamin A contents of materials which are not available for long periods of experimental feeding.

SUMMARY AND CONCLUSIONS.

Survival periods of rats of known age, pedigree, and previous nutritional history, when placed upon diet devoid of vitamin A but fully adequate in other respects, have been taken as measures of the relative stores of vitamin A which the body had accumulated.

By feeding diets graded in their content of vitamin A, it has been shown that the richer the diet in this vitamin the greater is the amount stored in the body. Rats of the same age but different dietary history survive the withdrawal of vitamin A from their foods in accordance with the richness of their previous diet in this vitamin; but the increase in the survival period is not arithmetically proportional to the increase in the level of vitamin A intake.

The bodily store of vitamin A was quickly and largely increased by the addition of cod liver oil to an already good diet; but the animal only gradually completes the process of acquiring the maximum quantity which the body is capable of storing.

Feeding of a normal diet enriched by the addition of 4 per cent of cod liver oil for 1 week just previous to the transfer of the animals to a diet devoid of vitamin A resulted in a striking prolongation of life of the animals. 2, 4, and 8 weeks of such feeding resulted in progressively greater prolongation of life but here also the increase in the survival period, which presumably measures the store of vitamin A which the body had accumulated, was not in arithmetical proportion to the length of time that the diet rich in vitamin A had been fed.

Attainment of the maximum store of vitamin A is apparently a process of gradual accumulation which is relatively rapid in its earlier stages and becomes slower as the maximum is approached. A rapid storage of the entire maximum amount is apparently not possible.

In these experiments, both when the level of intake of vitamin A was moderate and when it was very liberal, the animals continued to increase their bodily store throughout the entire period of rapid growth. Of the ages here studied, that of 6 months (in the rat) regularly showed the highest results when opportunities for storage had been equal. Given different opportunity for storage, however, it is easily possible for a 2 months old rat to obtain a reserve as great as that in a 6 months old animal of the same inheritance but of less fortunate dietary history.

A COMPARISON OF THE VISCOMETRIC, COPPER REDUCTION, POLARISCOPIC, AND IODOMETRIC METHODS FOR MEASURING THE RATE OF HYDROLYSIS OF STARCH AND DEXTRIN BY ASPERGILLUS ORYZÆ.

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(Received for publication, January 11, 1926.)

Comparison of the Viscometric and Copper Reduction Methods for the Study of Amylase.

The amounts of reducing substances produced by the action of 2 cc. of 0.1 per cent taka-diastase (in distilled water) upon 200 cc. of a boiled and autoclaved 6 per cent starch solution[1] at 34°C. and pH 5.7 were determined by removing 10 cc. samples at intervals, detroying the enzyme by boiling, and titrating the amount of copper reduced by the Bertrand method (1). Viscometric determinations (2) with the same concentrations of enzyme and substrate were made simultaneously. As illustrated in Fig. 1, the rate of the production of reducing substances was similar to that of the changes in viscosity.

The actual amounts of reducing sugar formed during the hydrolysis were comparatively small if expressed as maltose. For example at pH 5.7 (Fig. 1), the initial amount of copper reduced by 100 cc. of the starch-enzyme mixture was 115 mg. After 17 minutes of incubation at 34°C., 274 mg. were reduced. The amount progressively increased until after 24 hours it was 1340 mg. It is, of course, not definitely known whether the reducing substances were dextrin or maltose or a mixture of sugars. Sherman and Punnett (3) found that very little dextrose was formed

[1] 6 per cent of the particular lot of Lintner starch (10.1 per cent moisture) used was required to produce a viscosity two and a half times that of water.

during the digestion of 2 per cent starch by taka-diastase. If one assumed, that the reducing substance was maltose then, from Bertrand's table (1), the initial concentration of maltose in the starch-enzyme mixture would be 0.1 per cent. After 17 minutes of incubation, the amount would be 0.25 per cent and after 24 hours 1.2 per cent.

In the viscometric determinations the initial viscosity of 100 fell to 82 per cent in 17 minutes, to 69 per cent in 1 hour, and to

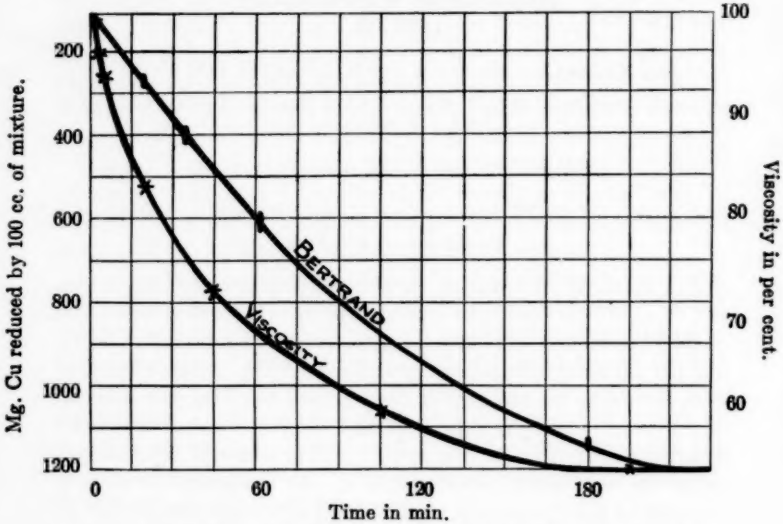


FIG. 1. The effect of 2 cc. of 0.1 per cent taka-diastase upon 200 cc. of a 6 per cent starch solution at pH 5.7 and 34°C. as determined by changes in viscosity and in the amount of copper reduced (Bertrand).

52 per cent in 24 hours. The viscosity of boiled and autoclaved 6 per cent solutions of Kahlbaum dextrin (8.3 per cent moisture) and bacteriological maltose (4.9 per cent moisture), which may or may not have the same viscosity as that of the dextrin and maltose produced during the hydrolysis of Lintner starch, was determined. On the same scale as that of Fig. 1 the viscosity of these solutions, taking into consideration the different water content of each, would be represented as 52 and 42 respectively. The viscosity of water

would be 37.8. In other words, the viscosity of the starch-enzyme mixture after 24 hours incubation was much higher than that of 6 per cent maltose and but slightly lower than that of a 6 per cent solution of Kahlbaum dextrin.

Comparison of Four Methods for the Study of Amylase.

In an effort to compare four methods of measuring the rate of the hydrolysis of starch (pH 5.7) by amylase, we added 2 cc. of a 0.1 per cent solution of taka-diastase (in distilled water) to 200 cc. of boiled and autoclaved 6 per cent starch solution, incubated the mixture at 34°C., and determined the changes in copper-reducing power, polariscopic rotation, and in the amounts of mixture required to produce a faint blue color with iodine after 30 minutes, 70 minutes, and 20 hours of incubation. Viscometric determinations with the same concentrations of enzyme and starch were made simultaneously. The reduction tests and viscometric determinations were performed as previously described. The polariscopic determinations were made by removing and boiling 15 cc. of the starch-enzyme mixture and then adding the sample to 1.5 cc. of M/5 Na_2CO_3 to stop any mutarotation present. The rotation of the mixture was then determined by using a 5 cm. tube in a Schmidt and Hänsch polariscope. Instead of the usual sodium flame, the light of a 125 watt Mazda bulb was employed. This was screened by placing a Wrattan filter, No. 73 (Eastman Kodak Co.), and a disc of gold glass (Bowen and King, Baltimore) in the distal aperture of the polariscope. By this means, practically the same spectrum as that of a sodium flame was obtained and the rotation of dextrose solutions was similar to that determined with a sodium flame.

The color tests with iodine were made by removing and boiling 5 cc. samples of the starch-enzyme mixture. After the end of the experiment, we determined the amount and the dilution of each sample which would produce a similar faint blue color when added to 5 cc. of Gram's iodine, previously diluted 1 to 125. We found that 0.2 cc. of a 1 to 10 dilution of the sample removed after 30 minutes of incubation produced the same faint blue color as 0.5 cc. of a 1 to 50 dilution of the original starch-enzyme mixture. Assuming that the starch-enzyme mixture before incubation contained 54 mg. of starch per cc. (6 per cent solution of starch con-

taining 10.1 per cent moisture), then 0.5 cc. of a 1 to 50 dilution contained 0.54 mg. As 0.2 cc. of a 1 to 10 dilution of the 30 minute sample produced the same color with iodine, this amount probably contained 0.54 mg. or 27 mg. per cc. After 70 minutes, 0.1 cc. of

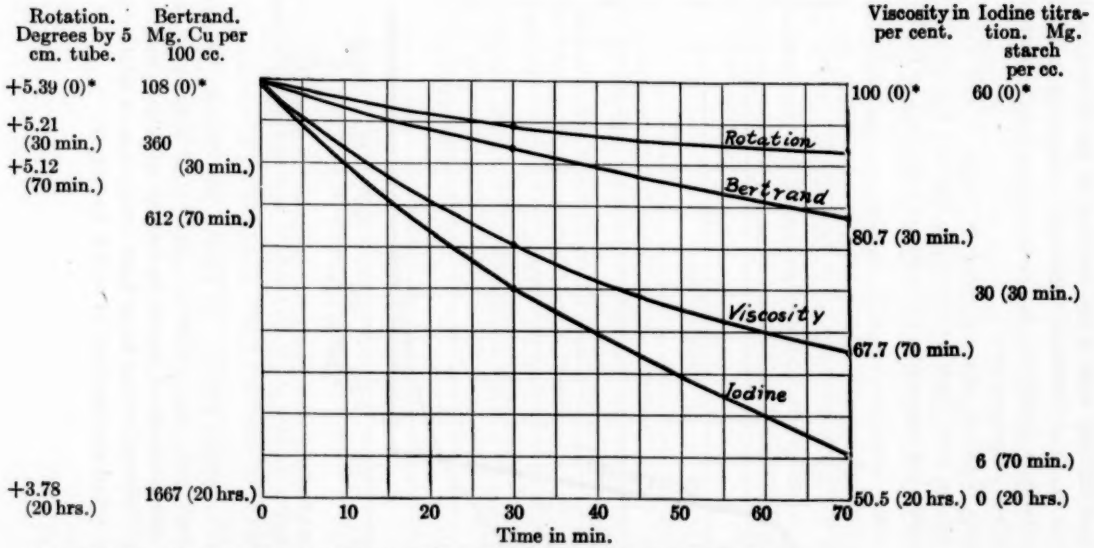
TABLE I.

Analyses of 5.4 Per Cent Starch Solution during Hydrolysis by Taka-Diastase and Analyses of 5.4 Per Cent Solutions of Starch, Dextrin, and Maltose (without Enzyme).*

Method.	Length of incubation at 34°C.				5.4 per cent starch.	5.4 per cent dextrin.	5.4 per cent maltose.
	0	30 min.	70 min.	20 hrs.			
Viscosity (in per cent of original viscosity).....	100	80.7	67.7	50.5	100	52	42
Bertrand (in mg. of Cu reduced by 100 cc. of solution).....	108	360	612	1667	108	638	6494
Rotation (in degrees of rotation by 5 cm. tube).	+5.39	+5.21	+5.12	+3.78	+5.39	+4.36	+2.85
Iodine titration (in mg. of starch per cc. of solution).....	54	27	5.4	0	54	0	0

* Since the starch employed contained 10.1 per cent moisture, the 6 per cent solution was really 5.4 per cent instead of 6 per cent. The 6 per cent dextrin and maltose solutions were 5.5 and 5.7 per cent respectively instead of 6 per cent, for the dextrin contained 8.3 per cent moisture and the maltose 4.9 per cent. In order that the viscosity, copper reduction, and polariscopic rotation values of the two latter solutions might be comparable to those of the starch solution, they were reduced to those of 5.4 per cent solutions; *i.e.*, the various values determined for the dextrin solutions were multiplied by $\frac{5.4}{5.5}$ or 0.98 and those obtained for the maltose solutions by $\frac{5.4}{5.7}$ or 0.95. This procedure is justifiable as the viscosity, copper reduction, and polariscopic rotation values of these substances are practically linear functions of concentration.

the undiluted starch-enzyme mixture produced the same color as 0.5 cc. of a 1 to 50 dilution of the original. Therefore, 0.1 cc. probably contained 0.54 mg. or 5.4 mg. per cc. After 20 hours, no blue color was obtained with iodine even though 5 cc. of the



*Elapsed time (in parentheses) after the addition of enzyme to the starch solution.

FIG. 2. The effect of 2 cc. of 0.1 per cent taka-diastase upon 200 cc. of a 6 per cent starch solution at pH 5.7 and 34°C. as determined by changes in rotation, copper reduction (Bertrand), viscosity, and iodine color.

starch-enzyme mixture were added to 5 cc. of the dilute Gram's iodine. This method, however, although frequently used in enzyme titrations, is usually not accurate and is not recommended for quantitative measurements.

The results obtained are expressed in Table I and Fig. 2. It may, of course, have been a coincidence that the curves of Fig. 2 are so smooth, but apparently the rates, as measured by the four

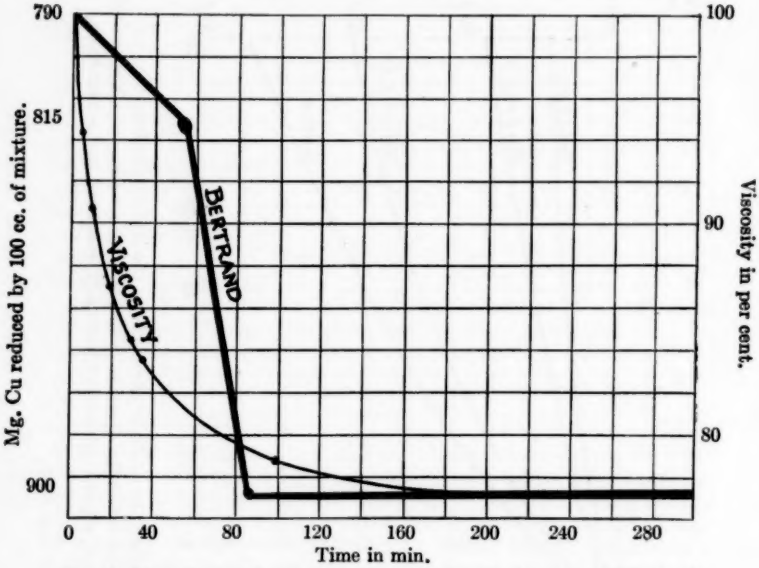


FIG. 3. The effect of 2 cc. of 10 per cent taka-diastase upon 100 cc. of a 15 per cent dextrin solution at pH 4.0 and 34°C. as determined by changes in viscosity and in the amount of copper reduced (Bertrand).

methods, are somewhat similar. In Table I, the viscosity, copper-reducing power, polariscopic rotation, and iodine titration of the theoretical end-products of starch hydrolysis are illustrated for comparison. We realize that these figures may bear no relation to those of the actual end-products of starch hydrolysis.

Comparison of the Viscometric and Copper Reduction Methods for the Study of Dextrinase.

In order to compare the Bertrand and viscometric methods of measuring the rate of hydrolysis of dextrin by taka-diastase, we added 2 cc. of a 10 per cent solution of taka-diastase (in distilled water) to 100 cc. of a boiled and autoclaved 15 per cent solution (pH 4.0) of dextrin (Kahlbaum), incubated the mixture at 34°C., and determined the changes in copper-reducing power after 55, 85, and 380 minutes of incubation. Viscometric determinations with the same concentrations of enzyme and dextrin were made simultaneously. The results obtained are expressed in Fig. 3. The rates of hydrolysis as determined by these two methods were somewhat similar.

CONCLUSIONS.

The rate of hydrolysis of starch solutions at 34°C. by taka-diastase was determined by viscometric, copper reduction, polariscopic, and iodometric methods. The rate of hydrolysis of dextrin solutions by taka-diastase was also measured by the first two of these procedures. The viscometric method is much the simplest of the four and it is probably as accurate as the others, for the curves plotted from the results obtained by each of these methods are somewhat similar.

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THE EFFECT OF THE HYDROGEN ION CONCENTRATION UPON THE STARCH-LIQUEFYING ACTIVITY OF THE AMYLASE OF ASPERGILLUS ORYZÆ.

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In order to determine by viscometric methods (1), the effect of the hydrogen ion concentration of starch substrates upon the activity of the amylase of *Aspergillus oryzæ* (taka-diastase), it was first necessary to ascertain the effect of acid and alkali alone upon the viscosity of starch solutions.

Effect of Hydrogen Ion Concentration upon the Viscosity of Unbuffered Starch Solutions.—10.2 gm. of commercial soluble starch (Merck), made according to Lintner's method (2), were thoroughly mixed in 500 cc. of distilled water, boiled for 1 minute, and then autoclaved at a pressure of 18 pounds for 15 minutes. After cooling to 34°C., the solution was made 500 cc. with distilled water and filtered through muslin. 49 cc. of this solution (containing 1 gm. of soluble starch) were placed in each of nine 50 cc. volumetric flasks. The amount of HCl or NaOH required to adjust the reaction of each flask to the desired pH was added and the total volume was made 50 cc. with distilled water. The approximate amounts of acid or alkali required are indicated in Table I. These amounts differ for different lots of starch. The hydrogen ion concentration was determined by colorimetric methods (3). The viscosity of each of these nine solutions was determined in large Ostwald viscosimeters at 34°C. with accurate stop-watches. These results are expressed in Table II in terms of relative viscosity; *i.e.*, the observed viscosity divided by the viscosity of water for the particular viscosimeter employed. A viscosity approximately two and a half times that of water is the most suitable for accurate measurements and this was produced

by a 2 per cent solution of the lot of starch employed. These boiled and autoclaved solutions of starch in water were colorless and 100 cc. would reduce only 127 mg. of copper (Bertrand method (4)). As this amount is equivalent to 0.1 per cent maltose or 0.07

TABLE I.

Approximate Amounts of HCl or NaOH Required to Adjust the Reaction of 50 Cc. of 2 Per Cent Boiled and Autoclaved Starch Solution to pH 1 to 9 Inclusive.

Desired pH of 2 per cent starch solution.	HCl	NaOH
1.0	0.66 cc. 10 N	
2.0	0.5 " N	
3.0	0.5 " N/10	
4.0	0.1 " N/10	
5.6	0	0
6.0		0.16 cc. N/100
7.0		0.166 " N/10
8.0		0.22 " N/10
9.0		0.43 " N/10

TABLE II.

Effect of the Hydrogen Ion Concentration upon the Viscosity of 2 Per Cent Starch Solutions at 34°C.

pH of solution.	Relative viscosity $\left(\frac{\text{observed viscosity}}{\text{viscosity of water}}\right)$.
1.0	2.22
2.0	2.27
3.0	2.41
4.0	2.64
5.6	2.61
6.0	2.55
7.0	2.57
8.0	2.59
9.0	2.58

per cent dextrose it is improbable that very much hydrolysis occurred as a result of this treatment. However, if starch is excessively heated with acid or alkali, hydrolysis and caramelization are produced. This made it necessary to cool the aqueous

solutions before acid or alkali was added. Inasmuch as it has been reported (5) that the addition of small amounts of various salts to substrates made with commercial soluble starch, which already contains a certain amount of salts, does not affect the saccharogenic activity of *Aspergillus oryzæ* (taka-diastase), salts were not added to our starch preparations.

As may be seen from Table II, reactions from pH 3.0 to pH 9.0 had but little effect upon the viscosity of starch solutions. At pH 1.0 and 2.0 partial precipitation of the starch occurred. This may perhaps explain the reduction of viscosity, for suspensions of undissolved starch have practically the same viscosity as water. The viscosity of starch solutions at pH 3.0 to pH 9.0 did not appreciably change after standing at 34°C. for 24 hours. The viscosity of the starch solutions at pH 1.0 and 2.0, however, decreased slightly after 24 hours, probably because of the precipitation which had very noticeably increased. It has since been found (6) that although the initial viscosity of solutions at pH 3.0 to 9.0 made with this same lot of starch after it had been kept at room temperature for several months had not changed, yet the viscosity was unstable and decreased progressively at 34°C. Solutions made with unstable starch are, of course, useless for the viscometric determination of enzyme action. The instability of the viscosity of the solutions made with this sample of starch did not depend upon the water content of the starch, for at the time the starch produced stable solutions it contained 9.2 per cent moisture, while 5 months later, when it was unstable, it contained 9.4 per cent. Other samples of starch which have been kept bottled for several months did not become unstable.

Effect of Hydrogen Ion Concentration of Unbuffered Starch upon the Starch-Liquefying Activity of Amylase (Taka-Diastase).—10 cc. of a 2.0 per cent starch solution at pH 1.0, prepared as described above, were placed in a large Ostwald viscosimeter at 34°C. and the initial viscosity determined. 0.1 cc. of a 0.2 per cent solution of commercial taka-diastase in distilled water was then added to the 10 cc. of starch solution in the viscosimeter, thoroughly mixed by shaking, and the time noted. The viscosity was determined at intervals of 3 or 4 minutes for a period of 1 hour. The viscosimeter was then cleaned with boiling water and dried with alcohol and ether. The same procedure was repeated with 2.0 per cent

starch solutions at pH 2.0, 3.0, etc. The hydrogen ion concentration was determined after the period of incubation and found to be the same as at the beginning of the experiment.

The results of the determinations are expressed in Fig. 1. The abscissæ represent the elapsed time (in minutes) after adding the enzyme. The ordinates represent the observed viscosities expressed as percentages of the initial viscosity (1). The viscosity

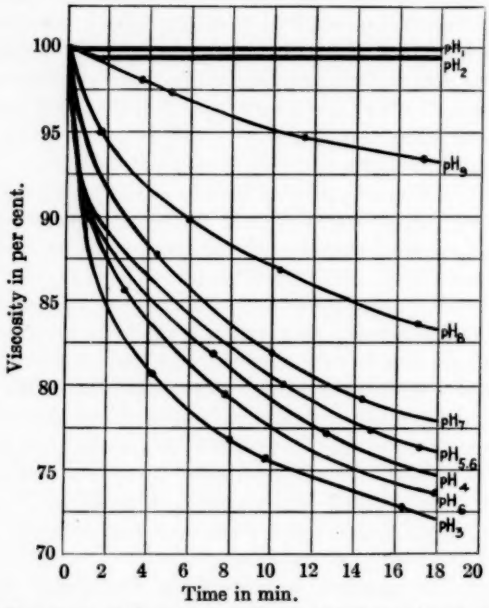

FIG. 1. The effect of the reaction of the substrate (2 per cent starch solution) upon the starch-liquefying activity of amylase (*Aspergillus oryzæ*, taka-diastase).

of starch solutions containing boiled inactivated enzyme remained constant for 24 hours or more. These curves represent the averages of four experiments done at different times in which the determinations at each hydrogen ion concentration were made in duplicate.

The effect of the hydrogen ion concentration of the starch solu-

tion upon the starch-liquefying activity of the amylase of taka-diastase, illustrated in Fig. 1, can also be expressed in numerical

TABLE III.

Effect of Hydrogen Ion Concentration of the Substrate (2 Per Cent Starch Solution) upon the Starch-Liquefying Activity of the Amylase of Aspergillus oryzæ at 34°C. in Units per Gm. of Dried Taka-Diastase (6.3 Per Cent Moisture).

pH of substrate.	Time required for 20 per cent reduction of initial viscosity by 0.1 cc. of 0.2 per cent taka-diastase in 10 cc. of substrate (Fig. 1).	Units per amylase per gm. of dried taka-diastase = $\frac{60}{T} \times \frac{10 \times 500}{0.937}$.
	min.	
1.0	∞	0
2.0	∞	0
3.0	4.7	68,121
4.0	9.1	35,183
5.6	10.6	30,205
6.0	7.1	45,094
7.0	12.6	25,410
8.0	5.6*	18,295
9.0	10.6†	4,229

* Indicates time required for 10 per cent reduction in initial viscosity. Units are calculated on basis of $\frac{19.2}{T}$.

† Indicates times required for 5 per cent reduction in initial viscosity. Units are calculated on basis of $\frac{8.4}{T}$.

units[1] per gm. of dried taka-diastase (Table III). For example, 0.1 cc. of 0.2 per cent taka-diastase required 4.7 minutes to reduce

[1] A unit of amylase is that amount of enzyme which requires 60 minutes to reduce the initial viscosity 20 per cent. It was found that if 0.1 cc. of an enzyme preparation required 60 minutes to reduce the initial viscosity 20 per cent, 0.2 cc. would reduce it a similar amount in 30 minutes. If the initial viscosity was reduced 20 per cent in 10 minutes, the enzyme preparation was assumed to contain $\frac{60}{10}$ or 6 units, etc. It has also been noted in a large series of amylase reactions that, except in those of great rapidity, the amount of enzyme which required 60 minutes to reduce the initial viscosity 20 per cent would reduce it 10 per cent in an average of 19.2 minutes and 5 per cent in 8.4 minutes. Therefore, if the reaction has been so slow that the initial viscosity has not been reduced 20 per cent during the period of observation, the number of units can be determined from the time required to produce a 10 or 5 per cent change.

the initial viscosity of 2 per cent starch at pH 3.0, 20 per cent (Fig 1). Therefore, 0.1 cc. of 0.2 per cent taka-diastase contains $\frac{60}{4.7}$ or 12.77 units. 1.0 gm. of the dried product (6.3 per cent moisture) would contain $12.77 \times \frac{10 \times 500}{0.937}$ or 68,121 units. Fig. 2 illustrates the effect of the hydrogen ion concentration of the substrate upon the starch-liquefying activity of amylase, expressed in units per gm. of dried taka-diastase.

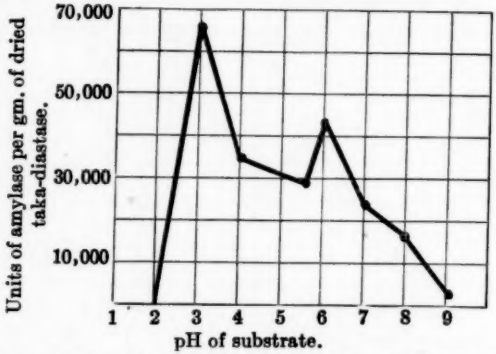

FIG. 2. The effect of the reaction of the substrate (2 per cent starch) upon the starch-liquefying activity of the amylase of *Aspergillus oryzæ*, in units of amylase per gm. of dried taka-diastase.

It is apparent from Figs. 1 and 2 that the optimal reaction for the starch-liquefying effect of taka-diastase upon a 2 per cent starch solution without additional buffer, as determined by viscometric methods, was at pH 3.0 and that the limits of activity were between pH 2.0 and pH 9.0. With an exception at pH 6.0, the activity of amylase decreased progressively from pH 3.0 to pH 9.0. We are unable at present to explain this exception at pH 6.0. At pH 1.0 and 2.0, the enzyme was completely destroyed, for the addition of sufficient alkali to readjust the reaction to the optimum failed to reactivate the enzyme. The exposure of taka-diastase for 5 minutes to sufficient HCl to produce a reaction of pH 1.0 and 2.0 also completely destroyed the amylase.

Effect of Hydrogen Ion Concentration of Starch Solutions containing Universal Buffer Solution upon the Starch-Liquefying

Activity of Amylase (Taka-Diastase).—In the preceding experiments no buffer was added because the hydrogen ion concentrations of the solutions of commercial Lintner starch were stable and did not change even after 20 hours hydrolysis. However, in order to study the effect of additional buffer, a boiled and autoclaved 4 per cent solution of starch was made. 25 cc. of this solution were added to each of six flasks containing 25 cc. of normal universal buffer solution (a mixture of phosphate, formate, acetate, sulfonate, and thymol) (7) at pH 2, 3, 4, 5, 6, and 7. The hydrogen ion concentration of the resulting 2 per cent starch solutions containing N/2 universal buffer solution were in each instance the same as that of the original normal buffer solution.

The rate at which 0.1 cc. of 0.1 per cent solution of commercial taka-diastase in distilled water reduced the initial viscosity of 10 cc. of each of these solutions was then determined as previously described. These determinations were made in duplicate on five different occasions. Reactions similar to those illustrated by the curves of Fig. 1 were obtained except that the rate of hydrolysis was the most rapid at pH 4.0 and that at pH 3.0 as well as at pH 2.0 the enzyme was destroyed. The relative rates at pH 4, 5, 6, and 7 were in the order named but the differences between the rates at these hydrogen ion concentrations were not as great as those illustrated in Fig. 1 for unbuffered starch solutions. Furthermore, the same amount of enzyme can apparently reduce the viscosity of starch solutions containing universal buffer more rapidly than that of unbuffered starch solutions. It is possible that one or more of the constituents of the universal buffer may act as an accelerator in this enzyme reaction.

DISCUSSION.

In viscometric determinations at 34°C. the optimal reaction for the hydrolysis of unbuffered starch solutions by the amylase of *Aspergillus oryzæ* (taka-diastase) was pH 3.0 and of starch solutions buffered with N/2 universal buffer pH 4.0. Of course, to determine the optimal reaction accurately, the hydrolysis of solutions whose hydrogen ion concentrations varied only $\frac{1}{10}$ of a pH should be studied. However, this change of the optimum from pH 3.0 to pH 4.0 after the addition of this buffer mixture was so striking that one is led to believe with Wilstätter and his co-

workers (8) that the optimal reaction for an enzyme instead of being specific for that enzyme may be dependent upon the chemical substances present in the enzyme-substrate mixture. He found that the optimum for a crude solution of lipase was from pH 5.5 to 6.3 and for a preparation purified with kaolin from pH 7.1 to 7.9. However, different pH optima with different buffers may not be real, for true hydrogen ion values are not always obtained by the ordinary methods of measurement.

The optimal hydrogen ion concentration for an enzyme reaction also depends upon the type of buffer solution used. Michaelis and Pechstein (9) found that the optimal reaction for saliva diastase was pH 6.1 to 6.2 in phosphate, acetate, and sulfate mixtures and pH 6.9 in chloride and nitrate mixtures. Hahn and Meyer (10) reported that the optimal pH for saliva diastase was 6.6 in a phosphate mixture and 5.6 in an acetate mixture, while the corresponding optima for pancreatic diastase were pH 7.2 and 5.6.

The optimal reaction for an enzyme also depends upon the temperature at which the hydrolysis is determined, for Compton (11) found that the amount of maltose converted by the maltase of *Aspergillus oryzæ* (taka-diastase) reached a maximum at pH 3.0 when determined at 32.5°C. and at pH 7.2 at 47°C. Sörensen (12) found that pH 4.4 to 4.6 was the optimum for yeast invertase at 52.1°C. and Michaelis and Davidsohn (13) reported that pH 4.2 was the optimum at 22.3°C. Olson and Fine (14) found that the optimal reaction for malt diastase was pH 4.3 at 25°C. and pH 6.0 at 69°C. These differences in optimal hydrogen ion concentrations at different temperatures may be fictitious as Thomas[2] suggested. In all the cases cited, the pH values were measured at room temperature but the true pH value at the temperature of the experiment may have been quite different.

It is possible that differences in the chemical content of the enzyme-substrate preparation as well as in the temperature at which the amylase activity was studied are responsible for the discrepancies between the optimum of pH 3.0 for taka-diastase which we found with unbuffered starch, pH 4.0 with buffered starch, and pH 4.8 reported by Sherman, Thomas, and Baldwin (15). Sherman, Thomas, and Baldwin added a solution of purified

[2] We wish to express our thanks to Dr. A. W. Thomas for many helpful suggestions.

taka-diastase to a 2 per cent solution of Lintner starch at various hydrogen ion concentrations and after 30 minutes of incubation at 40°C. determined the amount of reducing sugar with Fehling's solution. They found that the greatest enzymatic activity was at pH 4.8 and the limits were between pH 2.6 and 8.0.

Differences in the methods used may also have been responsible for the difference between our results and those of Sherman, Thomas, and Baldwin. The changes in the starch which cause a decrease in viscosity may not be the same as those which produce an increase in the reduction of copper. When two different methods are used to measure the activity of an enzyme, especially methods in which the rate of the disappearance of the substrate and of the formation of end-products is determined (as in diastatic enzymes), distinctly different optima may be observed. For instance, Sjöberg (16) reported that the optimal zone for the activity of *Phaseolus* diastase was at pH 4.0 to 6.0 if the rate of the disappearance of starch was measured but at pH 5.0 to 5.5 if the rate of the formation of reducing sugars was determined. Gore (17), with the polarimetric method, found practically the same optimal pH range for malt amylase which Sherman, Thomas, and Baldwin reported for this enzyme with the copper reduction method. Both of these methods measured the saccharogenic action of the enzyme while the viscometric method we used measured the starch-liquefying power.

Different criteria for enzyme activity may also be responsible for differences in results. We have adopted the time required to effect a definite amount of change in the substrate as our standard while others have determined the amount of change in the substrate in a definite period of time. It has been shown (1) that in viscometric determinations the former is proportional to the activity of the enzyme employed while the latter is not.

The optimal reaction for an enzyme apparently depends upon so many factors that it is not surprising that the optima for the digestive and other body enzymes as determined by laboratory methods are sometimes different from the reactions at which these enzymes must function in the body.

CONCLUSIONS.

1. The viscosity of 2.0 per cent starch solutions was only slightly affected by changes of reaction from pH 3.0 to 9.0. Starch was partially precipitated at pH 1.0 and 2.0.

2. The optimal reaction for the starch-liquefying activity of the amylase of *Aspergillus oryzæ* (taka-diastase) was at pH 3.0 and the limits of activity were between pH 2.0 and pH 9.0 when determined with unbuffered starch solutions by the viscometric method at 34°C. The optimum was at pH 4.0 when the starch solutions were buffered with N/2 universal buffer.

3. The amylase was completely destroyed at pH 1.0 and 2.0 with unbuffered starch and at pH 2.0 and 3.0 with starch buffered with N/2 universal buffer.

4. It is worthy of comment that the optimal pH for amylase both with unbuffered and buffered starch was close to the reaction at which the enzyme was destroyed.

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THE EFFECT OF THE HYDROGEN ION CONCENTRATION UPON THE DEXTRIN-LIQUEFYING ACTIVITY OF THE DEXTRINASE OF ASPERGILLUS ORYZÆ.

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Before studying the effect of the hydrogen ion concentration upon the activity of the dextrinase of *Aspergillus oryzæ* by viscometric methods (1) we determined the effect of acid and alkali alone upon the viscosity of dextrin solutions.

Effect of Hydrogen Ion Concentration upon the Viscosity of Dextrin Solutions.—76.5 gm. of dextrin (Kahlbaum) and 1.5 gm. of NaCl were thoroughly mixed in 420 cc. of distilled water, boiled for 1 minute, and then autoclaved at a pressure of 18 pounds for 15 minutes. After cooling to 34°C., the solution was made 500 cc. with distilled water and filtered through muslin. 49 cc. of this solution (containing 7.5 gm. of dextrin and 0.147 gm. of NaCl (50 millimols)) were placed in each of eight 50 cc. volumetric flasks. The amount of HCl or NaOH required to adjust the reaction of each flask to the desired pH was added and the volume was made 50 cc. with distilled water (Table I). The hydrogen ion concentration was determined by colorimetric methods (2).

A 15 per cent dextrin solution was used because its viscosity, approximately twice that of water, was the most suitable for accurate measurements; more dilute and more concentrated solutions were not as satisfactory. The dextrin solution was boiled and autoclaved for reasons given elsewhere (1) for the similar treatment of starch. 50 millimols of NaCl were added to the dextrin substrates because that amount had been found (3) to be optimal for the activity of taka-diastase when purified, relatively salt-free, starch substrates were used. The dextrin used contained 7.9 per cent moisture.

As may be seen from Table II reactions from pH 2.0 to 9.0 had but little effect upon the viscosity of dextrin solutions. The viscosity of these solutions did not appreciably change after standing at 34°C. for 24 hours.

TABLE I.

Approximate Amounts of HCl or NaOH Required to Adjust the Reaction of 50 Cc. of 15 Per Cent Boiled and Autoclaved Dextrin Solution to pH 1 to 9 Inclusive.

Desired pH of 15 per cent dextrin solution.	HCl	NaOH
1.0	0.35 cc. 10 N	
2.0	1.0 " N	
3.0	0.15 " "	
4.0		0.25 cc. N /10
5.0		0.75 " N /10
6.0		1.0 " N /10
7.0		0.25 " N
8.0		0.35 " "
9.0		0.5 " "

TABLE II.

Effect of the Hydrogen Ion Concentration upon the Viscosity of 15 Per Cent Dextrin Solutions at 34°C.

pH of solution.	Relative viscosity $\left(\frac{\text{observed viscosity}}{\text{viscosity of water}}\right)$.
2.0	2.17
3.0	2.19
4.0	2.21
5.0	2.22
6.0	2.22
7.0	2.22
8.0	2.28
9.0	2.16

Effect of Hydrogen Ion Concentration of the Substrate upon the Dextrin-Liquefying Activity of Dextrinase (Taka-Diastase).—10 cc. of a 15 per cent dextrin solution at pH 1.0, prepared as described above, were placed in a large Ostwald viscosimeter at 34°C. and the initial viscosity determined. 0.2 cc. of a 10 per cent solution

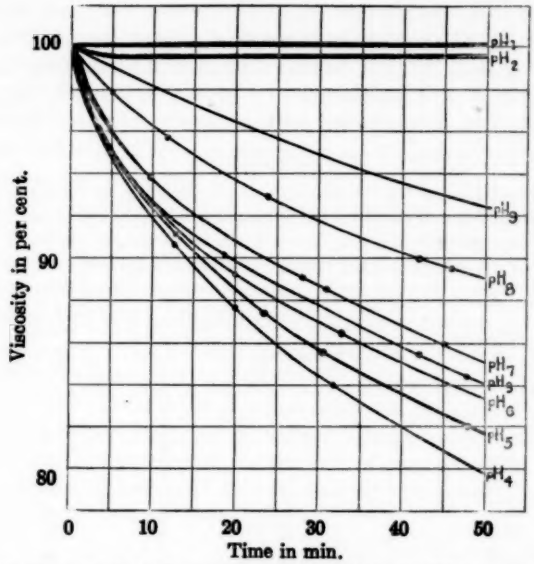

FIG. 1. The effect of the reaction of the substrate (15 per cent dextrin solution) upon the dextrin-liquefying activity of dextrinase (*Aspergillus oryzæ*, taka-diastase).

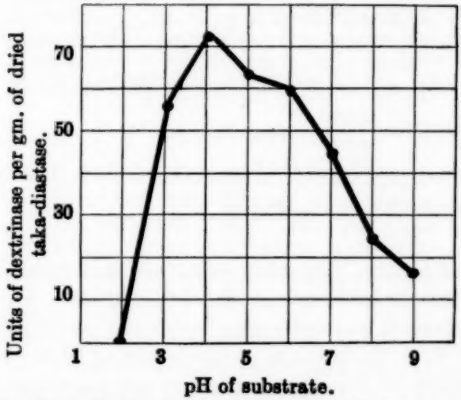

FIG. 2. The effect of the reaction of the substrate (15 per cent dextrin) upon the dextrin-liquefying activity of the dextrinase of *Aspergillus oryzæ* in units of dextrinase per gm. of dried taka-diastase.

of commercial taka-diastase in distilled water were then added to the 10 cc. of dextrin solution in the viscosimeter, thoroughly mixed by shaking, and the time noted. The viscosity was determined at intervals of 3 or 4 minutes for a period of 1 hour. The viscosimeter was then cleaned with boiling water and dried with

TABLE III.

Effect of the Hydrogen Ion Concentration of the Substrate (15 Per Cent Dextrin Solution) upon the Dextrin-Liquefying Acitivity of the Dextrinase of Aspergillus oryzæ at 34°C. in Units of Dextrinase per Gm. of Dried Taka-Diastase (6.3 Per Cent Moisture).

pH of substrate.	Time required for 10 per cent reduction of initial viscosity by 0.2 cc. of 10 per cent taka-diastase in 10 cc. of substrate (Fig. 1).	Units of dextrinase per gm. of dried taka-diastase $\frac{19.2}{T} \times \frac{5 \times 10}{0.937}$*.
	min.	
2.0	∞	0
3.0	18.7	55
4.0	14	73
5.0	16	64
6.0	17	60
7.0	23	45
8.0	42	24
9.0	28.5†	12

* For example, 0.2 cc. of 10 per cent taka-diastase required 18.7 minutes to reduce the initial viscosity of 10 cc. of 15 per cent dextrin at pH 3.0, 10 per cent (Fig. 1). Therefore, 0.2 cc. of 10 per cent taka-diastase contains $\frac{19.2}{18.7}$ or 1.03 units, and 1.0 gm. of the dried product (6.3 per cent moisture) contains $1.03 \times \frac{5 \times 10}{0.937}$ or 55 units.

† Indicates time required for 5 per cent reduction in initial viscosity. Units are calculated on the basis of $\frac{6.6}{T}$.

alcohol and ether. The same procedure was repeated with 15 per cent dextrin solutions at pH 2.0, 3.0, etc. The hydrogen ion concentration was determined after the period of incubation and found to be the same as at the beginning of the experiment.

The results of the determinations are expressed in Fig. 1, the abscissæ representing the elapsed time (in minutes) after adding

the enzyme, and the ordinates the observed viscosities expressed as percentages of the initial viscosity (1). It is apparent from Fig. 1 that the optimal reaction for the effect of taka-diastase upon a 15 per cent dextrin solution, as determined by the viscometric method, was at pH 4.0 and that the limits of activity were between pH 2.0 and 9.0. These experiments were repeated three times and similar results obtained.

The effect of the hydrogen ion concentration upon the dextrin-liquefying activity of the dextrinase of taka-diastase can also be expressed (Table III and Fig. 2) in numerical units per gm. of dried taka-diastase; *i.e.*, a unit of dextrinase being that amount of enzyme which requires 60 minutes to reduce the initial viscosity of the substrate 20 per cent, or 19.2 minutes to reduce the initial viscosity 10 per cent (1). The dextrinase reactions are relatively very slow so the number of units has been calculated upon the basis of a 10 per cent change. From a large series of dextrinase reactions it was found that the amount of enzyme which required 19.2 minutes to reduce the initial viscosity 10 per cent, required 6.6 minutes for a 5 per cent reduction.

CONCLUSIONS.

1. The viscosity of 15.0 per cent dextrin solution was but slightly affected by changes of reaction from pH 2.0 to pH 9.0.

2. The optimal reaction for the dextrin-liquefying activity of the dextrinase of *Aspergillus oryzæ* (taka-diastase) was pH 4.0 when determined by the viscometric method at 34°C.

3. The dextrinase was completely inhibited at pH 2.0.

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IS CYANIC ACID AN INTERMEDIATE PRODUCT OF THE ACTION OF UREASE UPON UREA?

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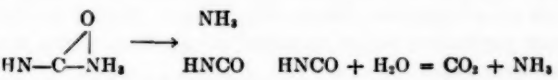
Having been engaged in a study of the purification and chemical nature of the enzyme urease for a number of years, I have naturally been interested in the hypothesis of Fearon that cyanic acid is formed as an intermediate product when urea is decomposed by urease.[1,2] Fearon believes that urea exists in solution as the cyclic form and that the action of urease consists in a removal of ammonia from the urea molecule, with the liberation of cyanic acid. He believes that the cyanic acid thus formed is spontaneously hydrolyzed to carbon dioxide and ammonia. The reactions are:



cyanic acid

Fearon offers the results of a number of experiments as evidence that cyanic acid is present during the zymolysis of urea by urease. One of these experiments consisted in adding a saturated solution of urea in absolute alcohol to soy bean meal that had been previously defatted with ether and extracted with absolute alcohol at 40°C. until the alcoholic extract no longer gave a precipitate with silver nitrate. After 11 days at 18°C. the maximum content of cyanic acid was observed. Cyanic acid was identified by its ability to give a precipitate with silver nitrate that did not dissolve upon bringing the medium to a pH of 5.0 with dilute nitric

[1] Fearon, W. R., *Biochem. J.*, 1923, xvii, 84.
[2] Fearon, W. R., *Biochem. J.*, 1923, xvii, 800.

acid. The precipitate could be decomposed by heating with nitric or hydrochloric acid and the ammonia formed by the hydrolysis of the cyanic acid could be estimated by Nesslerization.

Another type of experiment consisted in making a solution containing 0.6 per cent of urea and 0.1 per cent of urease (Dunning). Aliquots were removed from time to time and treated with an excess of silver nitrate. Dilute nitric acid was added in amount sufficient to bring the reaction of the test sample to a pH of 5.0, using a standard end-point and methyl red as indicator. This acidification had the object of decomposing any silver carbamate that might be present. The precipitate of silver cyanate was then washed until free from ammonia, digested on a water bath with 2 per cent nitric acid, and the ammonia formed by the decomposition of the cyanic acid aerated off and Nesslerized. Mack and Villars[3] had previously demonstrated the production of cyanic acid by the action of urease upon urea by a procedure almost identical with the above, but differing in the respect that they did not bring the reaction of the test sample to a pH of 5.0 to decompose any carbamate that might be present. Unlike Fearon, Mack and Villars did not interpret their results to mean that cyanic acid is an intermediate product of the zymolysis of urea, but considered the formation of cyanic acid to be the result of a side reaction. By a process of elimination Mack and Villars concluded that ammonium carbamate is the intermediate product of the action of urease upon urea.

I have repeated Fearon's experiment of allowing urease to stand in contact with a saturated solution of urea in absolute alcohol, using jack bean meal instead of soy bean meal. Inasmuch as jack bean meal is said to contain sixteen times more urease than soy bean meal one would expect that cyanic acid would be formed more rapidly than with the latter. However, no trace of cyanic acid was detected at any time. Aliquots were withdrawn, filtered, and tested for cyanic acid every day for 2 weeks and then every week for a period of 3 months. The experiment was repeated several times. Although the jack bean meal used in the experiment had been extracted many times with absolute alcohol at 40°C. the test portions that were treated with silver nitrate always gave

[3] Mack, E., and Villars, D. S., *J. Am. Chem. Soc.*, 1923, xlv, 505.

a very slight opalescence. This opalescence was no more marked in the solution containing the urea than in the control and was entirely insoluble in hot concentrated nitric acid. No ammonia was present at any time. After standing in absolute alcohol for 3 months the urease in the meal was still extremely active, although partly insoluble in distilled water.

I have attempted to repeat the experiment of Mack and Villars and of Fearon wherein urea is treated with urease in aqueous solution, using jack bean meal that had been extracted with petroleum ether and then with warm absolute alcohol as a source of urease. As a part of the precipitate with silver nitrate stayed in colloidal suspension, on account of the protein present, it was difficult to filter off and the experiment was unsatisfactory. In order to avoid the interfering action of impure urease preparations I have carried out some experiments with insoluble urease prepared by a modification of the method of Sumner, Graham, and Noback[4] and Sumner and Graham.[5] The insoluble urease employed is estimated to have been fully 100 times purer than the original jack bean meal and consequently something like 1600 times more active than soy bean meal. After the reaction the insoluble urease was removed by filtration and could be used for another experiment. In the experiments of Mack and Villars and of Fearon one part of urease preparation was used for every six parts of urea. In my experiments from 12 to 30 mg. of insoluble urease were added to 300 cc. of a 1.6 per cent urea solution. In order to prevent the possible decomposition of cyanic acid the solutions of urea were cooled to 10°C., or lower, before adding the urease and were at once placed in a room at from 2–4°C. After 4 hours the solutions were filtered and a 1 cc. sample was Nesslerized. The total production of ammonium nitrogen was from 330 to 500 mg. The remainder of the filtrate was immediately treated with 10 cc. of 10 per cent silver nitrate. A large excess of silver nitrate was avoided because silver cyanate is soluble in excess of silver nitrate. The material was then brought to a pH of 5.0 by the cautious addition of nitric acid. Normal nitric acid was added until the silver carbonate

[4] Sumner, J. B., Graham, V. A., and Noback, C. V., *Proc. Soc. Exp. Biol. and Med.*, 1924, xxi, 551.

[5] Sumner, J. B., and Graham, V. A., *Proc. Soc. Exp. Biol. and Med.*, 1925, xxii, 504.

began to dissolve, then tenth normal nitric acid was used. Great care was taken to avoid a local excess of acid by adding the acid drop by drop with constant rotation. The precipitate was then centrifuged off and washed with 0.05 per cent silver nitrate until the washings contained no more ammonia. The precipitate was then treated with 10 cc. of tenth normal hydrochloric acid and heated to 40–50° for 20 minutes. The solution was filtered from the silver chloride and washed into a 100 cc. volumetric flask and Nesslerized directly. No ammonia was present, indicating the absence of cyanic acid in the precipitate by silver nitrate.

In order to check up on the procedure I have added small quantities of potassium cyanate to portions of the enzyme-urea solution and have attempted to recover the nitrogen in the manner described above. When 1.7 mg. of potassium cyanate were added to 150 cc. of the enzyme-urea solution none was recovered. Silver cyanate is not completely insoluble in water and it was found that 1 mg. of potassium cyanate in 150 cc. of water gave no precipitate with silver nitrate, while 2 mg. did give a precipitate. Upon adding 10 mg. of potassium cyanate to 150 cc. of the enzyme-urea solution, 77 per cent was recovered by the analytical procedure. Both Mack and Villars and Fearon, in some cases, found greater concentrations of cyanate than the above.

Fearon has performed experiments which purport to show that urease is capable of adsorbing both urea and ammonia from aqueous solution. The enzyme preparation used in these experiments was soy bean meal that had been extracted with ether and then with absolute alcohol. From work performed in this laboratory we have been able to show that the jack bean contains at most no more than 0.4 per cent of urease. As soy bean meal contains about one-sixteenth as much urease as jack bean meal the percentage of urease in soy bean meal cannot be more than 0.025. With a concentration of urease of this order I believe that constituents of the soy bean other than urease should be taken into consideration when deciding just what is responsible for the adsorption of urea and ammonia.

The most important objection I have to the theory that cyanic acid is an intermediate product of the zymolysis of urea by urease has to do with the comparative stability of cyanic acid, or rather of salts of cyanic acid. If urease acted in a medium as

acid as tenth normal hydrochloric acid one might expect that cyanic acid would be decomposed almost as rapidly as formed. But urease acts best in a neutral medium. Any cyanic acid formed, no matter whether in the keto or enol form, would be immediately converted into a salt of whatever base might be present. I venture to predict that if cyanic acid were the intermediate product of the action of urease upon urea the analytical chemist using urease would have to multiply his results by two in order to obtain the correct result for urea nitrogen. An alternative would be to heat the analysis with a mineral acid before attempting to estimate the ammonia. I have prepared pure potassium cyanate from several samples by recrystallization from boiling 95 per cent alcohol and have satisfied myself that dilute solutions of potassium cyanate can be kept for several days at room temperature in neutral solution, in ammoniacal solution, and in the presence of different concentrations of phosphates from the pH of 6.8 to neutrality without an appreciable amount of decomposition. Upon heating to 40°C. hydrolysis takes place even in neutral solution.

The production of ammonium cyanate by heating urea in distilled water on a steam bath is good evidence of the comparatively slow rate of destruction of cyanate in alkaline solution. In my experiments where insoluble urease was allowed to react with urea the reaction of the medium became alkaline a few minutes after the addition of the urease.

THE ADSORPTION OF NITROGEN BY HEMOGLOBIN.

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(Received for publication, February 24, 1926.)

Our attention was drawn to the question of the solubility of nitrogen in hemoglobin solutions by some experiments involving the equilibration of solutions with carbon monoxide. Our preliminary experiments indicated that surprisingly large quantities of nitrogen were dissolved by hemoglobin solutions and further work revealed that the variations in the amount, with change in pressure, did not follow Henry's law. The solubility of nitrogen in hemoglobin is probably not in itself of any particular importance, but if the solubility of oxygen is complicated in a similar manner, this effect will need to be considered in interpreting the relationships involved in the combination of oxygen with hemoglobin. Having in mind such possible interpretations we decided to investigate the behavior of nitrogen more fully. This seemed particularly worth doing inasmuch as direct investigation of the adsorption or non-adsorption of oxygen by hemoglobin solutions is very difficult owing to the chemical combination which takes place. Since of the inert gases nitrogen is most like oxygen in its physical properties and particularly in regard to its ease of adsorption, there is some justification for assuming a relationship for oxygen, analogous to that found experimentally for nitrogen.

That nitrogen, unlike hydrogen, is more soluble in blood or hemoglobin solution than in water has long been known. Bohr (1) found that whereas the solubility of hydrogen in blood corresponded to the value calculated from its solubility in water and the water content of blood, nitrogen was over half again as soluble as would be predicted on the same basis. Buckmaster and Gardner (2) also found that nitrogen was abnormally soluble in blood and in recent years Van Slyke and his coworkers (3, 4) have reported values for nitrogen solubility only slightly lower than

Bohr's. The surprising observation of Bohr that the excess of nitrogen was only taken up in the presence of oxygen will be referred to again later.

The great difference between hydrogen and nitrogen would lead one at once to suspect adsorption as being responsible for the peculiar behavior of the heavier gas since hydrogen is the least adsorbed of all gases except helium (5). If the process is adsorption, the amount of excess nitrogen should be proportional to the hemoglobin content and related to the pressure in accordance with the Freundlich adsorption equation (amount adsorbed = aP^b). In this equation b is a fraction; this expresses the experimental fact that adsorption is relatively much larger at low pressures. No results were found in the literature dealing with the behavior of nitrogen and hemoglobin solutions at pressures below 1 atmosphere or over a sufficiently wide range to show striking deviations from Henry's law. Findlay (6) working with hemoglobin and pure nitrogen investigated the range from 1 to 2 atmospheres and interpreted the abnormalities which he found as due to adsorption, though his results as reported give a value to b in the Freundlich equation greater than 1.

We have studied a number of different hemoglobin solutions of different concentrations equilibrated with nitrogen at pressures from 740 down to 4 mm. and find the results are in accord with the adsorption isotherm. The significance of these results in connection with the oxygen-hemoglobin system is discussed in the concluding paragraphs of this paper.

EXPERIMENTAL METHOD.

Preparation of Hemoglobin Solutions.—Horse hemoglobin was used for all of the solutions except Nos. 7 and 8. The red blood corpuscles were washed three times with 1.5 per cent sodium chloride solution in a centrifuge. The washed corpuscles were treated with about an equal volume of distilled water, and usually shaken vigorously for several minutes with a layer of toluene to assist laking. This was followed by a final centrifuging. Solutions 7 and 8 were of cow hemoglobin which had stood for over a week in the ice box and become laked during this time; the corpuscles had therefore not been washed with sodium chloride solution.

Solutions 9, 10, 11 were of carefully purified horse hemoglobin.[1] Solution 9 was a portion of the mother liquor from the final crystallization and was isoelectric. We diluted it a little to prevent crystals forming. Solutions 10 and 11 were made from the crystals; they were dissolved in some of Solution 9 with a little distilled water added containing enough of concentrated buffer mixtures to make the solutions M/10 with respect to the buffer salts.

Equilibration of Solutions.—The solutions were placed in a tonometer and brought to room temperature, then evacuated until froth began to leave the tonometer. The gas mixture was then admitted to fill the tonometer to atmospheric pressure, and the tonometer was given 2 or 3 minutes of vigorous shaking before repeating the evacuation. In the final equilibration the pressure inside the tonometer was brought to a few mm. over atmospheric pressure to serve to expel the solution into the pipette. The final shaking was for 5 to 10 minutes. The tonometer was always pumped out before saturating even when air was used as the gas, in order to avoid the possibility of supersaturation. The shaking after each refilling increased the effectiveness of the procedure but it was found necessary to repeat the evacuation as many as eight times in order to get all detectable oxygen out of the solution when working with pure nitrogen.

Measurement of Nitrogen.—The analysis of the dissolved gases was done in a Van Slyke apparatus ((4) pp. 524–525). In the first experiments a 2 cc. pipette was used and the sample was washed in with 5 cc. of gas-free solution. In order to increase the quantities of nitrogen measured, a larger pipette was later used, and in the procedure finally developed, a 4 cc. sample was taken and washed in with 3 cc. of solution.

Particular care was taken to have gas-free solutions for absorbing CO_2 and oxygen, and to avoid leaks and the admission of tiny air bubbles when running in mercury. The solution used for absorbing oxygen contained in each 100 cc. of water, 16 gm. of sodium hydrosulfite, 6.6 gm. of sodium hydroxide, and 2 gm. of anthraquinone-beta-sulfonate, as recommended by Fieser (7). The quantity of this solution used varied somewhat but care was taken to be sure that absorption was complete, before taking the

[1] Kindly furnished by Dr. R. M. Ferry and Dr. E. J. Cohn of the Harvard Medical School.

manometer reading. The mercury was then raised and the nitrogen bubble expelled followed, of course, by some solution. The mercury was lowered almost the height of the barometer and if a bubble of gas made its appearance the expulsion was repeated. Enough of the solution in the cup was run back into the apparatus to equal that expelled and the pressure was again read with the

TABLE I.

Material used.	Hb concentration.	N_2 adsorbed in 1 cc. of solution.	N_2 adsorbed per gm. of Hb.
	gm. per 100 cc.	*cc.*	*cc.*
Solution 4 (horse Hb), undiluted	22.5	0.0047	0.021
" 4, diluted to two-thirds concentration	15.0	0.0031	0.020
Solution 4, diluted to one-third concentration	7.5	0.0017	0.023
" 12, undiluted	15.9	0.0041	0.026
" 12, diluted one-half	7.95	0.0021	0.026
" 5, laked with toluene	12.23	0.0034	0.028
" 6, after shaking with toluene	8.0	0.0023	0.029
" 6, same material laked with water	8.0	0.0011	0.013
" 7, cow Hb in water	13.6	0.0027	0.020
" 8, similar solution shaken with toluene	16.1	0.0039	0.021
Solution 7, M/10 with NaCl	12.8	0.0031	0.024
" 7, M/10 with phosphate buffer pH 6.12	12.8	0.0023	0.018
Solution 7, with phosphate buffer pH 6.98	12.8	0.0024	0.019
" 7, M/10 with borate buffer pH 8.41	12.8	0.0036	0.028
" 7, with borate buffer pH 9.08	12.8	0.0029	0.023
" 9, purified horse Hb, isoelectric	4.5	0.0015	0.034
" 10, similar material in phosphate buffer pH 6.12	8.0	0.0038	0.047
Solution 11, similar material in borate buffer pH 8.41	8.5	0.0037	0.044

meniscus at the 0.5 cc. mark. The decrease in pressure represented that due to nitrogen.

The combined oxygen was measured with somewhat less care as a means of determining the hemoglobin concentrations. In calculating how much of the dissolved nitrogen actually found was due to the water and how much to the hemoglobin, we assumed

that the water present would dissolve as much nitrogen as it was found to do in the absence of hemoglobin. When using buffered solutions comparison was made with blank determinations with buffer solutions of the same concentration. As the equilibration was done at room temperatures, which varied in some cases over 2° from that of the blank, allowance was made in these cases for the temperature differences.

In Table I are given the amounts of nitrogen adsorbed in cc. of gas measured at 0°, 760 mm., by 1 cc. of solution for various solutions when equilibrated with air at room temperature (about 23°C.). In the last column the amount adsorbed per gm. of hemoglobin is given. The calculation of the values in Table I is illustrated below in the case of some typical experiments. The measurements of the total quantity of nitrogen taken up by the solution were consistent to about 1 per cent and the adsorbed nitrogen to 5 to 10 per cent.

Typical Experiments (with Air).

The solutions were saturated with air at 23°C. and 4 cc. samples analyzed in the Van Slyke apparatus, the gas being measured at 0.5 cc. volume and the temperatures indicated.

Material.	Hb per 100 cc. (from O_2 capacity).	Nitrogen pressure in mm. of Hg.			Temperature.	Adsorbed N_2. $(A-B)\frac{F}{2}$.
		Total. (A)	Dissolved. (B)	Adsorbed. (A-B)		
	gm.				*°C.*	*vol. per cent*
Water.....	None.	75	75	None.	22.3	None.
Solution 9.....	4.5	82	71.5	10.5	21.0	0.16
" 9.....	4.5	81	71.5	9.5	21.0	0.15
" 5.....	12.23	87	65	22	23.5	0.34

The concentration of hemoglobin was found in the usual way from measurements of oxygen capacity. The pressure A was the change in manometer reading when the nitrogen was expelled. The pressure B due to dissolved nitrogen was calculated by multiplying the value of A found for water by the water content of the solution. (A-B) the pressure due to adsorbed nitrogen was converted into volumes per cent by multiplying by 0.5 F, where F is the factor given by Van Slyke for use when 2 cc. samples are

analyzed. For 21°C. the value of F is 0.0308. In the experiments with oxygen containing only about 1 per cent of nitrogen, we subtracted 3 mm. (found in experiments with water) as due to dissolved nitrogen.

DISCUSSION.

Inspection of Table I, particularly the last column, will show that the amount of nitrogen taken up in any solution depends on the hemoglobin concentration. The first five experiments, performed in order to test the effect of dilution, show this strikingly, as do the values for Solutions 5, 12, and 6 after treatment with toluene. On the other hand the amount of nitrogen adsorbed does vary with the nature of the solution. The highest values found were with the material which had been given the most rigid purification. The presence of salts and buffer mixtures seems to have an effect but no general statements could be made regarding it without further study.

The use of toluene produced a striking effect in some cases, particularly Solution 5, as compared with Solution 6. The fact that Solution 6 had laked on standing with water and yet gave a higher figure, comparable with Solution 5, when shaken and centrifuged with toluene, would seem to indicate that the latter acted by helping to remove some material which interfered with the nitrogen adsorption. That toluene vapor was not being measured was demonstrated by the fact that the addition of a few drops to an untreated solution did not change the value for adsorbed nitrogen.

The best test of whether the extra nitrogen is taken up by adsorption or not is to see whether the variations in amount with variations in pressure follow the adsorption isotherm. Expressing the equation in the logarithmic form: log (amount adsorbed) $= \log a + b \log P$. Obviously if one plots log (amount adsorbed) *vs.* log P, the result will be a straight line with slope equal to b.

In Fig. 1 are given the results obtained with Solution 5 (Curve I) and Solution 9 (Curve II) when they were equilibrated with air, and with oxygen-nitrogen mixtures containing about 9 and 1 per cent nitrogen. The results with Solution 5 are from early experiments where the consistency of check experiments was less. It is ob-

Log of nitrogen adsorbed (cc. per 1000 gm. of Hb).

vious that a straight line can be drawn through experimental points. The experiments with Solution 9 were done near the end of the series and show better agreement with each other and with the straight line relationship. The data for these solutions are given in Table II; the slope b computed over the range 585 mm. to the lower pressure is given in the last column. It is evident that the value of b is essentially constant.

It is particularly noteworthy that the exponent b in the adsorption equation seems to vary over rather wide ranges under differ-

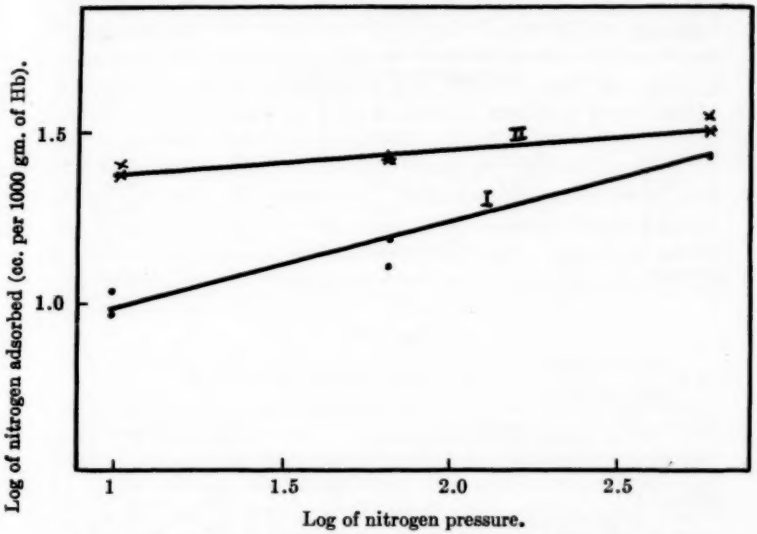

FIG. 1. Adsorption of nitrogen by hemoglobin at different pressures. Curve I, horse hemoglobin (Solution 5) unbuffered; Curve II carefully purified horse hemoglobin unbuffered and nearly isoelectric.

ent conditions and with different samples. A few experiments were performed to study the factors causing this variation. In most cases we assumed that the straight line relationship would hold and determined b from the slope of the curve between the points corresponding to air and a gas containing only a small amount of nitrogen. In some cases the low pressure of nitrogen was about 10 mm. or even less, while in some a pressure of about

TABLE II.

Nitrogen pressure.	N_2 adsorbed per gm. of Hb.	Log P.	Log N_2 adsorbed per 1000 gm. of Hb.	b
	Experiments with Solution 5.			
mm.	*cc.*			
585	0.0279	2.76	1.45	
66.6	0.0127	1.82	1.10	0.36
66.6	0.0151	1.82	1.18	0.28
9.6	0.0088	0.98	0.95	0.28
9.6	0.0113	0.98	1.06	0.22
Average.....				0.285
	Experiments with Solution 9.			
585	0.0359	2.77	1.55	
585	0.0325	2.77	1.51	
62.6	0.0271	1.79	1.43	0.11
62.6	0.0271	1.79	1.43	0.11
12.3	0.0237	1.08	1.37	0.09
12.3	0.0257	1.08	1.91	0.08
Average.....				0.10

TABLE III.

$b = \dfrac{\log P_1 - \log P_2}{\log C_1 - \log C_2}$, C_1 and C_2 being the amount adsorbed.

Material. (All horse Hb unless otherwise stated.)	Range of nitrogen pressure $P_1 - P_2$.		b
	mm.		
Solution 5.....	585 —	9.6	0.28
" 6, after toluene treatment.....	585	9.6	0.24
" 8, cow Hb after toluene treatment.....	585	9.6	0.22
" 7, cow Hb in water..	585	9.6	0.36
" 7, M/10 with NaCl.....	585	3.4	0.23
" 7, with buffer pH 6.12.....	585	9.6	0.39
" 7, " " " 6.98.....	585	9.6	0.33
" 7, " " " 8.41.....	585	3.4	0.43
" 7, " " " 9.08.....	585	3.4	0.40
" 9, unbuffered isoelectric purified Hb.....	585	12.3	0.10
Solution 10, similar to Solution 9, buffered pH 6.12 ..	585	62	0.30
" 11, " " " 9 " " 8.41 ..	585	62	0.31

60 mm. was used. The extra accuracy of measurements with the larger quantities of gas about offset the advantage of the greater range of nitrogen pressure.

In Table III are given the values of b found for different solutions of hemoglobin together with the range of nitrogen pressure over which it was found.

The data are too limited to enable one to draw definite conclusions but it is worth while to call attention to certain points in these experiments. The maximum variation of b with variation in material is between 0.1 and 0.4. The minimum value 0.1 was found with the most highly purified material, the adsorption measurements being checked at each of three pressures. This material was also practically isoelectric and salt-free, and the solution slightly more dilute than used in most of the other experiments. The addition of buffer mixtures to this material increased the value of b to a marked extent. With the cow hemoglobin the addition of buffers made little difference. With the buffers which we used the difference in pH made no very great difference in the value of b. The least value for the buffered solutions of cow hemoglobin was for a pH of 6.98 which is nearest the isoelectric point, but the difference is too slight to be of definite significance.

Adsorption of Nitrogen in the Absence of Oxygen.

All the experiments recorded so far were done with hemoglobin solutions saturated with air or with gas mixtures richer than air in oxygen, so that in all of them the hemoglobin was practically all in the form of oxyhemoglobin. The statement of Bohr that in the absence of oxygen no excess of nitrogen was taken up by hemoglobin solutions raised the question as to whether the adsorption of nitrogen was proportional to the oxyhemoglobin present rather than the total hemoglobin. We performed a large number of experiments with different hemoglobin solutions in which, by repeated evacuation and saturation with oxygen-free nitrogen, the total oxygen left in the solution was only 1 per cent or so of the saturation value. In most of these experiments a definite trace of oxygen was still found and enough nitrogen to correspond with the predicted solubility and adsorption at 740 mm. pressure. In the experiments where the oxygen content was too small to be detected with certainty (certainly much less than 1

per cent of the original) the nitrogen found was slightly less but still much greater than an equal volume of water would dissolve.

The studies which have been made on the simultaneous adsorption of more than one gas indicate that in general they do modify the behavior of each other. Some such effect may become detectable in the difference in the behavior of nitrogen with hemoglobin solutions after the latter have been given a prolonged evacuation. Our own experiments, however, did not confirm Bohr's statement that pure nitrogen is less soluble in hemoglobin solutions than in water, and we feel certain that the excess nitrogen taken up by hemoglobin solutions from oxygen-nitrogen mixtures depends on the concentration of total hemoglobin, not on the amount of oxyhemoglobin.

Adsorption by Hemoglobin as a General Phenomenon.

If the adsorption of nitrogen may be taken as evidence that hemoglobin has the general property of adsorbing gases, the implications of our results may extend far. We wish to call attention to two possible ways in which the phenomenon of adsorption may be of importance in the case of those gases which reversibly combine with hemoglobin. In the first place, in the experimental investigation of the oxygen-hemoglobin and carbon monoxide-hemoglobin system it is necessary to allow for "physically dissolved" gas in estimating the amount in chemical combination. If these gases, like nitrogen, are adsorbed on hemoglobin the correction must include not only gas dissolved in the water but also that adsorbed on the protein; at low pressures this correction may be considerable. In the second place, if the chemical combination is conditioned by an adsorption process, one would expect certain relationships which are very like those represented by the well known Hill equation. We will consider the question of correcting the experimental results for adsorbed gas before discussing the possible relation of Hill's equation to adsorption.

At the outset it should be emphasized that one could expect only a qualitative parallelism in the behavior of nitrogen and oxygen towards an adsorbing system. Thus if oxygen is adsorbed, we can confidently predict that the general equation $c_1 = aP^b$ will hold and that the values of b will be similar to but not identical with those found for nitrogen. The fluctuations in a

and b found in the case of nitrogen would be expected also with oxygen, but there would be no justification for assuming any quantitative parallelism. To illustrate the order of magnitude of the effects which may be expected, we shall use some of our nitrogen values in discussing oxygen but wish particularly to disclaim any idea that such figures may be made use of in a quantitative treatment of the oxygen system.

In Table IV we have illustrated the errors that would be introduced in the determination of the oxygen absorption curve *if oxygen were adsorbed to the same extent as nitrogen.* The data in the first three columns are similar to published figures for the equilibrium of 10 per cent solutions of horse hemoglobin with oxygen at 38°C., the dissolved gases being expressed as cc. per cc. of solution. The adsorption correction in Column 4 is calculated

TABLE IV.

P of O_2.	Total cc. of O_2 per cc. of solution.	Corrected for dissolved O_2.	Adsorption correction.	Corrected for adsorption.
348	0.1310	0.1212	0.0026	0.1186
104	0.1219	0.1189	0.0019	0.1170
37	0.0947	0.0936	0.0015	0.0921
18	0.0665	0.0661	0.0012	0.0649
8	0.0257	0.0255	0.0010	0.0245

by taking 0.25 as a typical value for b and 0.03 cc. per gm. of Hb as a typical value for the amount of gas adsorbed when the solution is shaken with air.

The actual corrections for oxygen may be greater or less, but the point which the table makes clear is that at low pressures the adsorption correction does not diminish nearly so fast as the correction for dissolved oxygen. Thus at 8 mm. pressure the adsorption correction is five times as large as the correction for dissolved oxygen. The smaller the value of b and the greater the amount of gas adsorbed the more striking will be the discrepancies found at low pressures. On the other hand it seems reasonable to suppose that spectroscopic methods will give the actual ratio of hemoglobin to oxyhemoglobin. We hope therefore to be able by a systematic comparison of the two methods at low pressures to study directly the adsorption of oxygen and carbon monoxide.

Chemical Combination of Gases with Hemoglobin.

The chemical equilibrium between hemoglobin and a dissolved gas must be conditioned by the effective concentration of the latter. If Henry's law is valid for the solutions of gases like oxygen and carbon monoxide in protein solutions, we are justified in substituting partial pressures directly in the mass law equations. If, however, oxygen and carbon monoxide like nitrogen are adsorbed by the protein it is extremely probable that this layer of adsorbed gas is concerned in the chemical equilibrium, and the effective concentration of oxygen is therefore not a simple function of the gaseous pressure. To account for the S-shaped saturation curves of oxyhemoglobin and carboxyhemoglobin systems, it has been assumed by Hill (8) that the hemoglobin molecule is reversibly associated in solution, and recently Adair (9) has sought to explain the complex phenomena in terms of a series of oxygen compounds. We should like to suggest a hypothesis differing from the others in that the peculiarities of the oxygen-hemoglobin systems are to be traced not to irregularities in the behavior of dissolved hemoglobin but to the adsorption of oxygen by the protein. This theory differs from the simple adsorption ideas of Ostwald (10) in that we imagine a definite chemical equilibrium *controlled by adsorption:* it may be regarded as an extension of the general viewpoint suggested by Bayliss (11) in his discussion of the problem.

The hypothesis, as we shall here present it, rests on the following assumptions: (1) that oxygen and carbon monoxide are adsorbed by hemoglobin; (2) that this adsorbed gas is involved in the chemical reaction between hemoglobin and oxygen or carbon monoxide; (3) that the hemoglobin molecule has a molecular weight corresponding to four atoms of iron per molecule and chemically combines with four molecules of oxygen; (4) that there is only one oxyhemoglobin (Hb_4O_8) and that intermediate compounds of the type Hb_4O_2, Hb_4O_4, etc., have no existence. The first two assumptions involve the basic principles of the point of view we are here presenting; aside from the analogy with nitrogen, they are at present unsupported by experimental evidence. The third and fourth assumptions are in accord with the most recent investigations of the molecular weight of hemoglobin (9, 12) and the spectrographic evidence which points to the existence of only one oxy-

hemoglobin. As will become evident in the discussion, the exact number of iron-containing complexes bound together in the hemoglobin molecule is not vital to the present argument and an equally satisfactory picture would result if the formula for oxyhemoglobin were taken as Hb_3O_6 or Hb_5O_{10}.

If we let Hb_4 represent the hemoglobin molecule and Hb_4O_8 oxyhemoglobin, we may write the following chemical reaction for the interaction of the protein and oxygen:

$$Hb_4 + 4\,O_2 \rightleftharpoons \underset{\text{(adsorbed)}}{Hb_4O_8}$$

When we imagine that there are no stable intermediate compounds between oxygen and hemoglobin and that no equilibrium exists involving even minute amounts of such compounds as Hb_4O_2, etc., it does not mean that such compounds may not have a transient existence. They may be formed from time to time, of course, during the interaction of oxygen and hemoglobin, but if they are we assume they are metastable and decompose *irreversibly* as illustrated below:

$$2\,Hb_4O_4 \rightarrow Hb_4O_8 + Hb_4$$

For such an assumption we have countless chemical analogies; for example, in the ammonia equilibrium, there are

$$N_2 + 3H_2 \rightleftharpoons 2NH_3$$

no side equilibria involving such hypothetical intermediates as N_2H_2, N_2H_4, etc. Similarly in the reversible oxidation of hydroquinone and stannous ion two hydrogen equivalents are transferred together.

The usual mass law equation for the chemical reaction written above would be:

$$\frac{\text{(hemoglobin)}\ (O_2\ \text{adsorbed})^4}{\text{(oxyhemoglobin)}} = K \qquad (1)$$

The adsorbed oxygen is related to the gas pressure by the expression $(O_2\ \text{adsorbed}) = aP^b$. Substituting this in (1) we obtain

$$\frac{\text{(hemoglobin)}\ P^{4b}}{\text{(oxyhemoglobin)}} = \frac{K}{a^4} = K' \qquad (2)$$

identical in form with Hill's equation, where $4b = n$ and K' is the usual constant. For a given solution of hemoglobin a is constant so that K' includes no variable term.

If the value of b were about 0.5, $4b = n$ would be about 2 as usually found with hemoglobin solutions, but would only by accident be exactly a whole number. The great advantage of the present hypothesis is that it gives a simple explanation of why the exponent n in Hill's equation is usually not an integer, and why the exponent is smaller than that predicted from the number of molecules of oxygen involved in the process, which on the basis of the new molecular weight of hemoglobin is almost certainly 4. Furthermore, if we may judge from the experiments with nitrogen, there might be some fluctuations in the exact value of n and K' depending on the source of hemoglobin, its method of purification, and variable factors in the solution. If the value of b under certain conditions dropped from 0.5 to about 0.25, n would be about 1 and the usual approximately hyperbolic curve would result. Since we cannot expect an exact quantitative parallelism in the behavior of nitrogen and oxygen in adsorption, it is not possible to put the present hypothesis on a satisfactory basis even if we were well informed as to the effect of all variables in the values of a and b in the nitrogen equation. Further work on the adsorption of nitrogen therefore seems to promise little help.

The hypothesis tells us nothing about the effect of such variables as the hydrogen ion concentration on the value of K or K' and leaves untouched those aspects of the oxygen-hemoglobin system which have been developed in this connection. Changes in the shape of the adsorption curve due to changes of K during the oxygenation are not involved in the present argument which applies strictly to buffered solutions. All that one can claim at present is that our experimental demonstration of the adsorption of nitrogen points to the possibility that a final explanation of Hill's equation may be found in terms of the adsorption of gases by the protein. A study of the adsorption of oxygen or carbon monoxide apart from the chemical combination of these gases—if such study proves possible—should either lead to the establishment of the ideas here presented or their definite rejection. In the meantime we venture to put forward this hypothesis since it serves to explain in simple terms some of the complicated relationships involved

in the combination of hemoglobin with oxygen and carbon monoxide.

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THE DETERMINATION OF URIC ACID IN BLOOD.

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For over 40 years following the introduction of Salkowski's (1) fundamental silver precipitation method for the determination of uric acid in blood, no substantial improvement was made until Folin and Denis (2) proposed their colorimetric procedure. Though the weak colors developed by their reagent were intensified by the use of potassium cyanide as suggested by Benedict and Hitchcock (3), yet the method was still laborious, requiring preliminary concentration of a deproteinized blood filtrate as well as large quantities of blood. The precipitation of uric acid directly from a dilute blood filtrate announced by Folin and Wu (4) in connection with their tungstic acid protein precipitant, therefore, marked a considerable advance in the uric acid determination, since but 2 cc. of blood were required and accuracy was increased.

This procedure was still further modified by Morris and Macleod (5) who, adapting a mode of uric acid precipitation previously suggested by Morris (6), eliminated one step in the Folin-Wu method, and made use of sodium cyanide as the only alkali in the color development as well as an arsenotungstic acid as the reagent.

Despite the seeming advantages of this method, the Folin-Wu procedure has, apparently, remained the accepted one until the introduction of Benedict's (7) direct modification. Owing to the simplicity of the latter and its use of much smaller amounts of blood, this technique has been widely adopted, particularly by clinical and hospital laboratories.

Benedict realized that his method gave high results in the region of the hitherto accepted normal range, and he assumed that owing to the extra manipulations required by the Folin-Wu method, some loss of uric acid probably occurs and that, therefore, the values obtained by the direct method should be more accurate.

For this reason he proposed that the upper limit of "normal" be raised about 1 mg. per 100 cc. However, it has been shown by Brown and Raiziss (8), Lennox and O'Connor (9), and Bulmer, Eagles, and Hunter (10) that such high results were due to the presence in blood of one or more unknown substances which produced a color with Benedict's reagent; and one such substance has recently been isolated (11).

Minor, though none the less important, disadvantages of the Benedict method are: First, exact timing and care in heating are essential; if the water bath is boiled too vigorously or the tubes allowed to remain in the bath too long color fading occurs. Second, turbidity develops at times so quickly that color comparisons must be hurriedly made at the sacrifice of accuracy, particularly if more than five determinations are made simultaneously. Dilution as Benedict (12) has pointed out will generally avoid this, but this expedient is not always efficacious and it can readily be shown that dilution is not the only factor in turbidity formation. Thus, in the Folin-Wu procedure the precipitates that occur during the color development usually appear first in the flasks containing the least amount of uric acid; and in the author's method described below, turbidity formation will be greatly accelerated if 1 instead of ½ cc. of reagent is used. As will be seen later, though the uric acid content per unit volume of solution is substantially the same in the author's and in Benedict's method, yet in the latter precipitates manifest themselves in about one-half the time.

That the Benedict reagent is not more specific for uric acid than the phosphotungstic acid reagent of Folin and Wu was early realized by us. In fact, none of the known uric acid reagents, of which there have been proposed at least five (13), can claim superiority due to specificity. In our efforts to modify the direct procedure in the hope of eliminating the faults noted above, we have prepared and tried a number of reagents including those already known. It was found that when used under identical conditions, the arsenotungstic acids give slightly more color with uric acid solutions than the phosphotungstic acids, but they are more easily affected by extraneous substances. For instance, boiling Benedict's reagent for an hour or more under the same conditions used in preparing the Folin-Wu reagent will produce in the former a much more highly colored solution.

Convinced that an increase in specificity for uric acid was not to be gained readily by a change of reagent, we directed our efforts to changing the conditions of the reaction for uric acid, using one reagent, *i.e.* the phosphotungstic acid proposed by Folin and Wu. In this we were aided by the observation of Benedict (14) that the uric acid reaction carried out in the cold produces a color development which is proportional in both standard and unknown. We had previously noted that rabbit blood tungstic acid filtrates gave more color when heated than when unheated. Seemingly, the substance isolated by Hunter and Eagles (11) does not react with the reagent readily in the cold in the presence of excess alkali cyanide. Since it is practically impossible to buy molybdenum-free sodium tungstate[1] (15), we endeavored to obtain the color development with but 0.5 cc. of the reagent. Theoretically 0.5 cc. should be sufficient for a relatively large amount of uric acid, and actually this amount is used in the Morris and Macleod method. It was hoped that the use of the smaller quantity of reagent would reduce the amount of color formed due to the action of the blood phenols. Further, the tendency to turbidity formation is lessened with reduction in the amount of reagent.

The above considerations finally led to the adoption of the following method.

Standard Solutions.—The stock lithium urate solution preserved with formaldehyde as recommended by Folin (13) is used because of its stability. From it a standard solution for use is obtained by diluting 2.5 cc. of the stock in a 500 cc. volumetric flask to 400 cc. with water, adding 1 cc. of formalin and 10 cc. of $\frac{2}{3}$ N sulfuric acid, and diluting to the mark. 5 cc. of this solution are equivalent to 0.025 mg. of uric acid and the hydrogen ion concentration corresponds closely to that of a properly made tungstic acid filtrate. Only 1 cc. of formalin instead of 2 is used as recommended by Folin, because apparently more formaldehyde retards the color development. A 1:500 solution of formalin is bacteriostatic at room temperature and the diluted standard will keep at least 8 weeks.

The *reagent* used is the phosphotungstic acid recommended by

[1] J. T. Baker Co.'s "special" sodium tungstate contains but minute amounts of molybdenum.

Folin and Wu (4) because of its simplicity of ingredients and as stated above its relative inertness to foreign substances. With the use of clean glassware and a good reflux condenser even 1½ hours gentle boiling will produce at most but a very light colored solution.

A 5 per cent *sodium cyanide*[2] solution was used which under the conditions given below gave absolutely no color in blank determinations.

10 cc. of tungstic acid blood filtrate are transferred to a 100 cc. Erlenmeyer flask[3] and 5 cc. of distilled water added. To two other flasks are added respectively 5 and 10 cc. of the diluted uric acid standard and the volumes made up to 15 cc. with water. To each flask is then added (from a burette) 5 cc. of 5 per cent sodium cyanide, and 0.5 cc. of the reagent, and the contents of the flasks are mixed. After 20 minutes standing at room temperature the usual colorimetric comparisons are made.

In this as in all other colorimetric determinations it is advisable to compare the unknown with a standard of nearly equal strength. The above two standards will take care of uric acid values up to about 6.5 mg. per 100 cc. of blood, which covers the more commonly occurring amounts. If higher results are expected a third standard may be prepared containing 15 cc. of diluted standard. For those rare occasions when the uric acid content is above 9 mg. it is best to repeat the determination using 5 cc. of filtrate, making up to 15 cc. with water and proceeding as above.

After 20 minutes standing at room temperature there is but little increase in strength of color and such increase is proportional in both the unknown and standard. Even with a large excess of sodium oxalate[4] present, turbidity formation in less than 40 minutes has not been encountered, and, since the colors do not fade for a relatively long period, it is evident that a large number of simultaneous determinations may be made—a distinct advantage in the busy hospital laboratory.

Further, the colors produced even with the smaller amounts of

[2] J. T. Baker's C.P. sodium cyanide.

[3] Test-tubes of adequate capacity may, of course, be used.

[4] Potassium ions in moderate amounts do not shorten the time of turbidity formation. The use of potassium oxalate as anticoagulant should, however, be discouraged.

uric acid are sufficiently intense so that no difficulty is met with in making comparisons.

The *calculations* are the usual ones: With the standard cup at 20 mm., if 5 cc. of standard are used the result in mg. of uric acid per 100 cc. of blood will be obtained by dividing the unknown

TABLE I.

Results Obtained by the Author's Method Compared with Those Obtained by the Folin-Wu and Benedict Methods on the Same Samples of Animal Blood.

Sample No.	Animal.	Uric acid per 100 cc. of blood.		
		Folin-Wu method.	Author's method.	Benedict method.
		mg.	*mg.*	*mg.*
1	Rabbit 1	0.0	0.6	
2	" 2	0.0	1.2	
3	" 3	0.0	1.0	
4	" 4	0.2	1.6	4.4
5	" 5	0.5	2.5	
6	" 6		2.9	5.9
7	" 7		2.8	6.0
8	" 8	0.5	2.2	5.1
9	" 9		2.6	4.9
10	" 10		2.6	4.7
11	Sheep 1	0.0	0.5	4.0
12	" 2	0.0	1.4	
13	" 3	0.0	1.0	
14	" 4	0.0	2.0	4.4
15	" 5	0.0	1.8	4.5
16	Dog 1	0.0	0.8	
17	" 2	0.0	0.7	
18	" 3	0.3	0.9	
19	" 4	0.5	2.0	5.0
20	" 5	0.0	1.8	4.2
21	Chicken 1	13.0	9.8	
22	" 2	3.4	4.0	
23	" 3	4.2	5.0	
24	" 4	4.8	5.2	

reading into 50; if the 10 cc. standard is used divide the reading into 100; if the 15 cc. standard is used divide into 150.

DISCUSSION.

Table I represented what may be expected of the author's technique with animal blood as compared with the Folin-Wu and

Benedict methods. Of those animals known to contain at most but a few tenths of a mg. of uric acid in 100 cc., high results are

TABLE II.

Recoveries of Uric Acid Added to Various Animal Blood Filtrates Obtained by the New Modification.

Sample No.	Type of blood.	Per 100 cc. of blood.				
		In original.	Added.	Total found.	Recovered.	Error.*
		mg.	mg.	mg.	mg.	mg.
1	Rabbit.	2.5	2.5	4.1	4.1	+1.6
		2.5	5.0	5.8	5.8	+0.8
		2.5	7.5	7.7	7.7	+0.2
		2.5	10.0	9.0	9.0	−1.0
2	"	1.0	2.5	3.0	3.0	+0.5
		1.0	5.0	5.3	5.3	+0.3
3	"	0.6	2.5	3.2	3.2	+0.7
		0.6	5.0	5.7	5.7	+0.7
		0.6	7.5	7.2	7.2	−0.3
		0.6	10.0	9.9	9.9	−0.1
4	Dog.	0.9	2.5	2.4	2.4	−0.1
		0.9	5.0	5.0	5.0	0.0
		0.9	7.5	7.7	7.7	+0.2
		0.9	10.0	9.6	9.6	−0.4
5	Human.	2.0	2.5	4.4	2.4	−0.1
		2.0	5.0	6.6	4.6	−0.4
		2.0	7.5	8.8	6.8	−0.7
6	"	2.2	2.5	4.4	2.2	−0.3
		2.2	5.0	6.9	4.7	−0.3
		2.2	7.5	9.4	7.2	−0.3
7	"	2.4	2.5	4.9	2.5	0.0
		2.4	5.0	7.5	5.1	+0.1
		2.4	7.5	9.0	6.6	−0.9

*The error calculated on the animal bloods is based on the assumption that they contain no uric acid; *i.e.*, the error indicates the effect of interfering substances on the uric acid determination.

obtained by both direct methods. However, the great increase in specificity by permitting the uric acid reaction to take place at

TABLE III.

Results Obtained by the Author's Method Compared with Those Obtained by the Folin-Wu and Benedict Methods on the Same Samples of Human Blood.

Sample No.	Uric acid per 100 cc. of blood.					Non-protein nitrogen.
	Folin-Wu method.	Author's method.	Benedict method.	Difference between.		
				Folin-Wu and author's methods.	Folin-Wu and Benedict methods.	
	mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
1	5.5	5.3		−0.2		50
2	6.0	5.9		−0.1		42
3	8.5	7.8		−0.7		180
4	6.0	6.0		0.0		37
5	4.1	4.1		0.0		30
6	5.0	4.7	5.8	−0.3	+0.8	27
7	4.4	4.2	6.0	−0.2	+1.4	30
8	4.3	4.5	7.2	+0.2	+2.9	26
9	3.2	3.3		+0.1		30
10	10.0	9.1	9.6	−0.9	−0.4	146
11	3.4	3.6		+0.2		27
12	4.0	4.0		0.0		29
13	3.7	3.9		+0.2		29
14	2.6	2.9		+0.3		35
15	4.7	4.2		−0.5		35
16	2.8	3.0		+0.2		35
17	3.5	3.9		+0.4		33
18	3.0	3.4		+0.4		28
19	6.3	6.3		0.0		39
20	3.2	2.6		−0.6		30
21	2.8	2.9	3.9	+0.1	+1.1	34
22	4.0	4.2	5.0	+0.2	+1.0	33
23	4.9	4.5	5.3	−0.4	+0.4	37
24	3.8	3.6		−0.2		33
25	4.2	4.8		+0.6		
26	2.5	2.3		−0.2		36
27	3.0	3.3		+0.3		32
28	3.3	3.4		+0.1		28
29	2.2	2.6		+0.4		30
30	2.1	2.2		+0.1		26
31	3.3	3.3		0.0		30
32	3.0	3.5		+0.5		30
33	3.1	3.4		+0.3		26
34	1.8	1.8	3.0	0.9	+1.2	26

TABLE III—*Concluded.*

Sample No.	Uric acid per 100 cc. of blood.					Non-protein nitrogen.
	Folin-Wu method.	Author's method.	Benedict method.	Difference between.		
				Folin-Wu and author's methods.	Folin-Wu and Benedict methods.	
	mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
35	2.1	2.3		+0.2		24
36	3.6	3.4		−0.2		29
37	2.5	2.4		−0.1		30
38	1.7	1.8		+0.1		27
39	2.4	2.2		−0.2		26
40	2.2	2.2		0.0		30
41	1.9	2.1		+0.2		29
42	2.4	2.4		0.0		29
43	3.6	3.7		+0.1		28
44	2.6	2.5		−0.1		33
45	2.9	2.9		0.0		35
46	2.8	2.8		0.0		35
47	2.0	2.0		0.0		30
48	2.1	2.2		+0.1		32
49	3.5	3.3		−0.2		34
50	3.5	3.0	4.0	−0.5	+0.5	28
51	3.5	3.7	5.0	+0.2	+1.5	35
52	5.4	5.0	5.4	−0.4	0.0	33
53	2.5	2.8	4.5	+0.3	+2.0	26
54	2.2	2.7	4.7	+0.5	+2.5	30
55	1.2	1.3	3.0	+0.1	+1.8	32
56	2.6	2.7	4.7	+0.1	+2.1	28
57	2.8	2.8	5.2	0.0	+2.4	28
58	2.0	2.2	3.2	+0.2	+1.2	32
59	2.5	2.5	3.5	0.0	+1.0	35
60	5.8	5.4	5.9	−0.4	+0.1	68
61	6.9	6.5	6.9	−0.4	0.0	109
62	3.0	2.5	4.0	−0.5	+1.0	
63	3.6	3.3	4.3	−0.3	+0.7	
64	3.5	2.8	4.3	−0.7	+0.8	
65	2.8	2.5	3.8	−0.3	+1.0	
66	2.8	2.8	4.4	0.0	+1.6	
67	4.9	4.1	5.1	−0.8	+0.2	
68	4.4	4.2	4.8	−0.2	+0.4	
69	3.5	3.5	4.0	0.0	+0.5	
70	3.6	3.6	4.7	0.0	+1.1	31
71	3.4	3.3	4.4	−0.1	+1.0	39
72	4.8	4.3	4.9	−0.5	+0.1	31
73	5.0	5.1	5.1	+0.1	+0.1	34
74	3.9	4.0	4.8	+0.1	+0.9	35

room temperature is clearly apparent from a comparison of the results obtained by the author's and Benedict's methods. Of interest are the results with chicken blood. The first chicken blood examined showed an unexpectedly high uric acid content and the result does not compare favorably with that obtained by the Folin-Wu method. Unfortunately, not enough blood was available to repeat the determination using 5 cc. of filtrate; however, the results with the other three chicken bloods are in better agreement.

Table II lists some typical recoveries of uric acid added directly to the tungstic acid filtrates of different bloods. The results with the animal bloods illustrate the extent of the interfering action of non-uric acid substances on the true uric acid content of the blood. The recovery experiments with human blood filtrates are interesting mainly in indicating that correct results for bloods containing more than 9 mg. per 100 cc. can only be expected if but 5 cc. of filtrate are used. It would appear that there is nothing present in human blood which inhibits the color development by the author's method.

Table III presents a comparison of the results obtained by the author's, the Benedict, and the Folin-Wu methods on the same tungstic acid filtrates of a miscellaneous series of human bloods.[5] The Folin-Wu method has, apparently, been accepted as a standard for comparison. Results by this technique are probably correct and, since any shorter method to be acceptable must be capable of giving similar values, it was at first deemed sufficient to include only results by this and the author's method. Only an occasional determination was, therefore, made by Benedict's method. However, it was thought advisable, later, to make determinations by all three methods in order that the increased specificity of the author's method might be more clearly emphasized.

A gratifyingly close agreement is seen to exist in the table between the author's and the Folin-Wu methods in all values ranging from 6.3 mg. down. In only six cases is the difference greater than 0.5 mg. and in no case is the difference more than

[5] Most of the bloods were obtained from patients in the practice and clinic of Dr. Jay F. Schamberg during the course of some other work being done with him.

1 mg. As was to be expected, higher results are consistently shown by Benedict's method, especially in the range of the hitherto accepted normal values. Evidence (16) is accumulating to show that such high results are incorrect. Such results are often misleading inasmuch as a 5 mg. per 100 cc. value (all three methods show fair agreement with bloods giving about this value by the Folin-Wu technique) would be of little significance if the normal limit be taken at 4.5 mg., whereas a definite pathological condition may actually exist. Such values are common and the actual conditions would be distinctly indicated only by the use of Folin and Wu's or the author's method.

Somewhat lower results apparently will be obtained by the author's method, when the blood contains large amounts of uric acid. Only two such cases were available to us and in both instances lower results were obtained. Benedict's method also has the same effect and his explanation (17) that such lower values are nearer the true ones is unsatisfactory to us. However, the question is purely an academic one. Obviously it is of more importance, practically, to be certain that results of 3 or 5 mg. are correct than results of 9 or 11 mg. It is worthy of emphasis here that high results are not obtained in the usually accepted normal range, and it is, therefore, unnecessary to assume a higher normal limit for uric acid in blood—an important point in diagnosis.

CONCLUSIONS.

A direct method for determining uric acid in blood is described which has the following advantages over previous direct methods:

1. The technique is simplified by permitting the uric acid reaction to take place at room temperature and thus eliminating heating in the water bath.

2. Turbidity formation is greatly delayed, making possible the simultaneous determination of a larger number of bloods.

3. Results are obtained which are nearer those of the Folin-Wu technique and, therefore, its use does not make necessary a new conception of higher "normal" uric acid values.

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STUDIES ON ENZYME ACTION.

XXXIV. ACTIONS OF SOME ESTER-HYDROLYZING ENZYMES AT DIFFERENT TEMPERATURES.

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INTRODUCTION.

The influence of increase in temperature on an enzyme action is taken to involve two factors; first, an increased action obeying the general rule that the velocities of all chemical reactions are increased, and second, a decreased action because of the inactivation of the enzyme. Because of the opposing nature of these two influences, an optimum temperature has been found for almost all enzyme actions between 35° and 50°.

In this paper some enzyme actions will be described which show considerably greater values at low temperatures (5–17°) than at the more usual optimum temperature (37°).

EXPERIMENTAL.

Methods.

Three kinds of enzyme material were used; eels, trout and trout eggs, and castor beans. The eels, trout, and trout eggs were obtained from the State Fish Hatcheries, Cold Spring Harbor, Long Island, New York. The results presented here form part of a more extended investigation with these materials. Thanks are due Mr. Stanley C. Walters, Superintendent of the Hatcheries for his constant cooperation and helpfulness in providing the materials as they were needed. Thanks are also due the State Conservation Commission for permission to use the fish and eggs

of the Hatcheries in this investigation. The castor beans were supplied by the Baker Castor Oil Company of New York.

The different natures of the materials used may serve to indicate that the phenomena observed are not limited to one special source of enzymes but are of quite general relevance. The enzyme actions studied in this connection involve the hydrolysis of certain comparatively simple esters. The advantages of studying these enzyme actions lie first, in the simple compositions of the substrates, which make the chemical changes studied very definite, second, in the simplicity of the estimations of the changes, and third, in the possibility of varying the substrate so that many different tests are practicable and small differences in the characters of the enzyme actions readily observable. Limiting the esters to those soluble in water eliminates one of the most troublesome factors in enzyme studies, although the fact that fats and other naturally occurring insoluble esters are not used narrows to a certain extent the applicability of some of the results.

In the enzyme tests, the material was passed several times through a meat grinder and definite quantities weighed and tested for enzyme action, or extracted with water or different solutions overnight, filtered through paper, and portions measured for the tests. In general, 15 cc. of water or extract and 3.4 or 6.8 milliequivalents of ester were used. Incubation was allowed to proceed for different lengths of time either in incubators at 37–38° or 55–60° or in running water for the low temperatures. The acid produced was estimated by titration with 0.1 N sodium hydroxide solution with phenolphthalein as indicator. Duplicates were run in every case and toluene was present throughout. The esters tested were the same as those studied in previous communications.[1]

Eels.—The results obtained with eels are shown in Table I. The natures of the mixtures tested are shown in Column 2 of this table, the temperatures of the incubations in Column 3. The concentrations of the mixtures were equivalent to 44.4 mg. of original substance per cc. of solution tested. The times of incubation were 22 hours; the original pH, 7.0. The enzyme

[1] Falk, K. G., Noyes, H. M., and Sugiura, K., *J. Cancer Research*, 1921, vi, 285; 1925, ix, 105; *J. Biol. Chem.*, 1922, liii, 75; 1923, lv, 653; lvi, 903; 1924, lix, 183, 213, 224; 1924–25, lxii, 687, 697; *J. Am. Chem. Soc.*, 1924, xlvi, 1885; *J. Gen. Physiol.*, 1925, viii, 75.

actions on 3.4 milli-equivalents of the different esters are given in the last ten columns in terms of cc. of 0.1 N sodium hydroxide solution (as actually measured) corrected for blanks, or as tenths of milli-equivalents of ester hydrolyzed by the enzyme material under the conditions of the experiments.

The actions on phenyl acetate were larger in every case at the lower temperature. The absolute differences varied with the different solutions, being greatest with the 50 per cent glycerol solution and smallest with the 10 per cent sodium chloride solution. With the other esters the actions were larger at the higher temperature, the differences varying also with the ester and with the solvent. The smallest differences in actions at the two temperatures were observed with glyceryl triacetate, the actions however being larger at the higher temperature except with the 10 per cent sodium chloride solution where they were practically the same at the two temperatures. These actions were readily reproducible at different times.

Trout.—The actions on the trout preparations are shown in Table II. Trout of different ages were used. One experiment with eggs is given. The headings of the columns are self-explanatory. The actions were tested for 22 hours, starting at pH 7.0, on 3.4 milli-equivalents of each ester, at the concentration of 44.4 mg. of original material per cc. of final solution tested.

The different factors to be considered in studying these results include the differences in the substrates, the differences in the extracting solutions, the differences in the ages of the trout, and the differences in the temperatures of testing the enzyme actions. With the water extract (first six pairs of results) the actions on the eggs were larger at the higher temperature with all the esters; with the 6 months trout, larger action on the phenyl acetate at the higher temperature, slightly larger on the glyceryl triacetate, but practically the same on the remaining esters at the two temperatures. For the 1½ years trout, the first series showed larger action at the lower temperature on all the esters, but especially on benzyl acetate, the second series showed either the same or somewhat larger actions on all the esters at the higher temperatures except for benzyl acetate where the action was somewhat larger at the lower temperature. The 2½ years trout gave definitely larger actions at the lower temperature on benzyl acetate, iso-

TABLE I.

Actions of Eel Preparations on the Indicated Esters in Tenths of Milli-Equivalents of Acid Produced.

Experiment No.	Mixture tested.	Temperature.	PhOAc	Gl(OAc)$_3$	MeO·COPr	PhCH$_2$OAc	EtOAc	MeOAc	EtO·COPr	MeOBu	EtOBu	i-BuOAc
		°C.										
F 18A	Solid, water.	15–17	5.38	2.64	1.67	0.86	0.67	0.83	1.27	0.22	0.11	0.76
F 18A	" "	37–38	4.50	4.07	3.01	1.36	0.93	1.00	2.10	0.57	0.32	1.42
F 15A	Water extract.	15–17	5.58	3.69	2.19	0.97	0.71	0.84	1.37	0.35	0.23	1.04
F 15C	" "	37–38	4.72	4.48	3.80	1.73	1.24	1.42	2.76	0.60	0.84	1.96
F 17B	" "	15–17	4.82	2.72	1.68	0.69	0.49	0.64	0.98	0.20	0.18	0.86
F 17B	" "	37–38	3.55	3.02	2.64	1.50	0.63	1.00	2.07	0.53	0.37	1.53
F 16B	" " dialyzed.	15–17	3.59	2.10	1.20	0.38	0.23	0.31	0.65	0.09	0.07	0.37
F 16D	" " "	37–38	3.07	2.58	2.20	0.95	0.61	0.74	1.62	0.26	0.24	1.00
F 17D	50 per cent glycerol extract.	15–17	5.77	2.44	1.51	0.65	0.54	0.67	0.96	0.21	0.12	0.68
F 17D	50 " " " "	37–38	3.70	3.25	2.59	1.20	0.78	1.01	1.95	0.48	0.30	1.34
F 17D	50 " " " "	15–17	2.64	1.25	0.76	0.26	0.12	0.17	0.59	0.04	0.03	0.22
F 17D	dialyzed.	37–38	2.29	1.50	1.32	0.63	0.36	0.41	0.95	0.22	0.19	0.61
F 19B	50 per cent glycerol extract.	15–17	6.64	2.60	1.67	0.65	0.49	0.58	1.06	0.23	0.11	0.70
F 19B	50 " " " "	37–38	3.83	3.63	2.97	1.17	0.79	1.03	2.10	0.40	0.33	1.41

F 19B	50 per cent glycerol extract	15–17	5.29	2.88	1.68	0.68	0.40	0.52	1.05	0.15	0.07	0.69
F 19B	dialyzed *vs.* distilled water, 6 hrs.	37–38	3.44	2.98	2.77	1.21	0.74	0.89	2.09	0.35	0.33	1.33
F 19A	10 per cent NaCl extract.	15–17	1.81	1.61	1.31	0.77	0.63	0.81	1.10	0.29	0.25	0.75
F 19A	10 " " " "	37–38	1.61	1.54	1.39	0.95	0.75	0.91	1.28	0.53	0.39	1.03
F 19A	10 " " " " di-	15–17	2.59	1.70	1.20	0.48	0.37	0.40	0.84	0.18	0.14	0.46
F 19A	alyzed.	37–38	2.41	1.79	1.52	0.85	0.63	0.74	1.29	0.37	0.29	0.95

TABLE II.

Actions of Trout Preparations on the Indicated Esters in Tenths of Milli-Equivalents of Acid Produced.

Experiment No.	Material.	Mixture tested.	Temperature.	PhOAc	Gl(OAc)$_3$	MeO·COPr	PhCH$_2$OAc	EtOAc	MeOAc	EtO·COPr	MeOBu	EtOBu	i-BuOAc
			°C.										
F 22A	Eggs removed from brown trout.	Water extract.	14	1.30	1.04	0.04	0.10	0.10	0.20	0.02	0.04	0.00	0.06
			37–38	2.42	1.79	0.13	0.18	0.29	0.39	0.11	0.07	0.05	0.19
F 13A	Brown trout 6 mos. old.	" "	17	1.47	0.73	0.53	0.51	0.35	0.41	0.48	0.07	0.08	0.51
			37–38	2.16	0.87	0.56	0.51	0.38	0.46	0.49	0.18	0.11	0.56
F 12E	Male brook trout 1½ yrs. old.	" "	16–18	0.78	0.61	0.23	0.54	0.19	0.29	0.17	0.13	0.14	0.32
			37–38	0.57	0.40	0.18	0.17	0.16	0.20	0.10	0.06	0.00	0.10
F 12B	Female brook trout 1½ yrs. old.	" "	16–18	0.59	0.31	0.20	0.42	0.16	0.17	0.11	0.20	0.21	0.18
			37–38	0.73	0.51	0.26	0.25	0.18	0.45	0.24	0.13	0.05	0.14
F 21D	Female trout 2½ yrs. old.	" "	15–17	1.37	0.63	0.43	0.61	0.20	0.27	0.22	0.14	0.10	0.41
			37–38	1.05	0.64	0.20	0.20	0.15	0.25	0.24	0.14	0.06	0.19
F 22C	Female brown trout 4 to 5 yrs. old.	" "	14	0.86	0.60	0.25	0.27	0.16	0.25	0.14	0.04	0.02	0.28
			37–38	1.25	0.96	0.32	0.23	0.13	0.18	0.14	0.02	0.00	0.21
F 10	Male rainbow trout 2½ yrs. old.	3 per cent NaCl extract.	15–17	1.32	1.09	0.32	0.93	0.35	0.77	0.16	0.00	0.00	0.79
			37–38	1.11	0.77	0.29	0.40	0.28	0.36	0.25	0.16	0.19	0.32

F 13B	Brown trout 6 mos. old.	10 per cent NaCl extract.		17	1.83	1.09	0.57	0.65	0.66	0.71	0.43	0.11	0.06	0.70
				37–38	2.06	1.11	0.56	0.48	0.38	0.52	0.32	0.10	0.06	0.56
F 12C	Female brook trout 1½ yrs. old.	10 " " " "		16–18	0.82	0.84	0.25	0.78	0.32	0.65	0.09	0.01	0.09	0.84
				37–38	0.75	0.61	0.13	0.27	0.15	0.25	0.17	0.09	0.25	0.17
F 13C	Brown trout 6 mos. old.	50 " " glycerol extract.		17	1.62	0.95	0.60	0.51	0.37	0.45	0.47	0.10	0.05	0.53
				37–38	2.22	1.05	0.65	0.48	0.41	0.49	0.49	0.23	0.11	0.59
F 12D	Female brook trout 1½ yrs. old.	50 " " " "		16–18	0.99	0.83	0.49	0.52	0.37	0.47	0.41	0.06	0.09	0.53
				37–38	0.92	0.49	0.34	0.22	0.17	0.26	0.18	0.06	0.09	0.20
F 5H	Rainbow trout 2 yrs. old.	50 " " " "		15–17	2.52	1.46	1.02	1.06	0.73	1.08	0.63	0.11	0.05	1.07
				37–38	2.58	1.74	1.18	0.98	0.67	0.72	0.90	0.31	0.17	0.84
F 11C	Female rainbow trout 2½ yrs. old.	50 " " " "	*	15–17	1.16	0.69	0.36	0.55	0.28	0.44	0.26	0.09	0.02	0.42
				37–38	0.93	0.52	0.12	0.24	0.16	0.19	0.07	0.07	0.07	0.10
F 11D	Female rainbow trout 2½ yrs. old.	50 " " " "	†	15–17	1.13	0.73	0.36	0.51	0.21	0.38	0.21	0.05	0.01	0.53
				37–38	1.10	0.64	0.28	0.31	0.26	0.33	0.26	0.25	0.12	0.23

* Stood at room temperature (25°) 18 hours before addition of esters and incubation.

† Stood at 15–17° 18 hours before addition of esters and incubation.

butyl acetate, and methyl butyrate, while the 4 to 5 years trout gave larger actions on phenyl acetate and glyceryl triacetate at the higher temperature and practically the same actions at the two temperatures on the other esters. The behavior of the 4 to 5 years trout in this respect was similar to that of the 6 months trout.

The extracts with the different solvents gave essentially the same relations. With the 3 per cent sodium chloride extract, considerably greater actions were found on benzyl, methyl, and isobutyl acetates. With 10 per cent sodium chloride solution the results on the trout of different ages were similar to the water extract. Larger actions were observed at the low temperature, strikingly with benzyl acetate and isobutyl acetate, somewhat less so with methyl acetate, and to a still smaller extent with the other acetates.

With the 50 per cent glycerol extracts, similar relations were observed. For the 6 months trout, greater action was obtained at the higher temperature on phenyl acetate, practically the same actions at the two temperatures on the remaining esters; for the 1½ years trout, larger actions at the lower temperature on all the esters except the benzoates; for the 2 years trout not much difference for the two temperatures except that ethyl acetate and isobutyl acetate were somewhat larger at the lower, glyceryl triacetate and methyl butyrate somewhat larger at the higher temperature. The last two experiments showed the effect of allowing the extracts to stand before testing the enzyme actions. The differences were small, it is true, but distinct, the actions of the extract which stood at the lower temperature being slightly greater throughout in the tests incubated at 37–38°.

The results obtained with the trout were less uniform than those obtained with the eels. This was partly due to the different ages of the trout as well as possibly to the different varieties. The results of the ester-hydrolyzing tests are sufficiently clear, however, to show some fundamental differences. With eels, the enzyme actions were greater, under the conditions used, on phenyl acetate at the lower temperature. With trout, if greater action was found at the lower temperature, it was on benzyl acetate and isobutyl acetate, then on methyl acetate, and rarely on phenyl acetate. It is true that a number of additional experiments was performed on trout some of which gave larger results throughout

at the higher temperature and some of which gave larger results at the lower temperature, but these will not be given as they add nothing to the data already presented.

Because of the larger action at the lower temperatures especially on benzyl acetate, some experiments with aqueous trout extract were carried out in which the concentration and the times of action were varied. These results (Experiment F 4) are shown in Table III.

With 6.8 m.-eq. of ester at 37–38°, the reaction appears to have stopped at 6½ hours. This seemed to be true also with 3.4 m.-eq. of ester at 37–38° and the more concentrated extract. At the lower temperature the reaction appeared to continue. The

TABLE III.

Hydrolyzing Actions on Benzyl Acetate in Tenths of Milli-Equivalents of Acid Produced by Aqueous Trout Extract.

Time.	3.4 m.-eq. of ester.		6.8 m.-eq. of ester.		Mg. of substance per cc. of solution.
	15–17°	37–38°	15–17°	37–38°	
hrs.					
6½	0.94	0.46	1.11	1.05	88.9
22	1.17	0.75	1.29	0.95	88.9
48	1.29	0.58	1.43	0.97	88.9
6½	0.41	0.08	0.67	0.52	44.4
22	0.49	0.30	0.68	0.36	44.4
48	0.75	0.33	0.91	0.42	44.4

actions were greater at the lower temperature in every case, but especially so with the smaller concentration of ester. It is inadvisable to draw more general conclusions on the basis of these few results.

The protease actions of extracts of trout of different ages were also studied at the two different temperatures. A peptone, a casein, and a gelatin preparation were used as substrates. The actions were measured by the formol method as described in previous papers.[2] Without entering into the results in detail, the general conclusion may be given that in every case the action

[2] *Cf.* Falk, K. G., Noyes, H. M., and Sugiura, K., *J. Biol. Chem.*, 1922, liii, 75.

was found to be greater at the higher temperature than at the lower.

Castor Beans.—Several preparations of castor beans were used. A method of obtaining the material was developed which is simpler and just as satisfactory as the former methods. Cold pressed castor beans were ground in a small meat grinder without removing the hulls, and extracted in an Erlenmeyer flask overnight with petroleum ether. The material was then air-dried and sifted through a 20 mesh sieve. The material was then extracted with ether in a Soxhlet apparatus for several days. On putting the air-dried, defatted material through a 60 mesh sieve, the remaining particles of hull were eliminated and there resulted a hull-free, fat-free castor bean preparation. This was used either in the form of weighed portions of the solid preparation to which water was added, or as aqueous or 10 per cent sodium chloride extracts (dialyzed and undialyzed).

The following results (Experiment 65) were obtained with weighed portions of castor bean preparation, 0.3 gm., in 15 cc. of water, acting on 3.4 milli-equivalents of the different esters for 24 hours, starting at pH 7.0, at the indicated temperatures. The actions, as usual, are given as cc. of 0.1 N sodium hydroxide solution used, corrected for blanks, or in terms of tenths of milli-equivalents of esters hydrolyzed by the castor bean preparation under the given conditions.

	PhOAc	Gl(OAc)$_3$	MeO·COPr	$PhCH_2OAc$	EtOAc	MeOAc	EtO·COPr	MeOBz	EtOBz	i-BuOAc
14.5–16°	7.44	1.15	0.38	0.33	0.46	0.50	0.30	0.00	0.02	0.17
37–38°	5.18	3.63	1.70	1.04	1.14	1.21	1.18	0.24	0.13	0.75

These results show greater actions on phenyl acetate at the lower temperature; but with all the other esters unmistakably greater actions at the higher temperature. The following experiment (No. 66) was carried out with the aqueous and sodium chloride extracts, undialyzed and dialyzed, of the castor bean preparation. The results are given in the same terms as those just shown, each cc. of extract tested corresponding to 20 mg. of original castor bean preparation. The 10 per cent sodium

chloride extract was prepared with the residue from the aqueous extract. The results obtained with four esters are given.

	°C.	PhOAc	Gl(OAc)$_3$	MeO·COPr	EtO·COPr
Water extract undialyzed	14	0.91	0.18	0.14	0.13
	37.5	0.82	0.47	1.05	0.90
Water extract dialyzed	14	0.96	0.12	0.05	0.08
	37.5	0.53	0.32	0.91	0.78
NaCl extract undialyzed	14	1.30	0.49	0.07	0.01
	37.5	0.88	0.72	0.02	0.02
NaCl extract dialyzed	14	0.67	0.16	0.00	0.00
	37.5	1.02	0.55	0.04	0.02

These results show again the differences in the actions on phenyl acetate and the other esters at the two temperatures. The larger actions on phenyl acetate at the lower temperature are more striking with the salt extract than with the water extract, although even with the latter there is a definite difference, especially with the dialyzed extract. The higher result on phenyl acetate at the higher temperature with the dialyzed sodium chloride extract is of interest and of possible significance.

The actions after different time intervals with phenyl acetate were studied at the different temperatures. The results of Experiment 68 are shown in Table IV. In this experiment, separate (and duplicate) portions of 0.1 gm. of castor bean preparation in 15 cc. of water were used with 3.4 milli-equivalents of phenyl acetate. The mixtures were titrated after standing for the indicated times at the different temperatures. Toluene was present throughout. The results, corrected for blanks, are given in the usual terms. Two sets of results are shown, in one the mixtures were brought originally to pH 5.0, in the other to pH 7.0.

The results at the two hydrogen ion concentrations did not differ greatly. Comparing the actions at 12° and 37°, the values

were somewhat greater at the higher temperature for the smaller time intervals. After 6 hours, the values at 12° increased steadily while those at 37° increased only slowly, so that the former from being only slightly larger at 6 hours became almost three times as large at 48 hours. The results do not form an exact curve. This is to be expected because of the manner of carrying out the determinations. Each result represents the mean of two separate measurements, so that the cumulative effect of errors introduced if portions of a common large amount of mixtures were used, are avoided, although at the same time irregularities were shown in

TABLE IV.

Time-Action Results with Castor Bean Preparation and Phenyl Acetate at Different Temperatures.

Time.	pH 5.0			pH 7.0		
	12°	37°	55°	12°	37°	55°
hrs.						
2	0.27	0.27	0.25	0.20	0.31	0.29
4	0.61	0.70	0.26	0.47	0.58	0.40
6	1.14	0.92	0.31	0.96	0.83	0.47
8	1.20	1.07		1.12	1.11	
12	2.50	1.31		2.38	1.35	
16	3.13	1.79	0.60	2.87	1.76	0.78
20	2.69	1.64		3.42	1.73	
24	3.98	1.85	0.66	4.54	1.81	0.90
28	5.41	2.21		5.53	2.32	
32	5.51	1.95		6.08	2.37	
40	5.81	2.53		6.48	2.40	
48	7.25	2.70	1.21	7.90	2.82	1.54

the individual results. In other similar series, the actions at the earlier times were larger at the higher temperature than those shown in this experiment, but the changes of the relative magnitudes of actions at 12° and 37° occurred, in general, between 6 and 8 hours.

The interesting feature of the results at 55° is the considerable amount of action still observable. This was found a number of times. Apparently part of the enzyme acting on phenyl acetate is comparatively heat-stable. In other experiments, the action found at 55° was distinctly greater than the actions found at 12° and 37° for the first 2 or 3 hours.

In order to attempt to separate the effects of temperature and other possible factors in these enzyme actions, a number of experiments were carried out in which the castor bean preparation was mixed with water, different portions allowed to stand at 5–7° and at 37° for various lengths of time and then both sets tested for 24 hours at the two temperatures after the addition of the esters. Enzyme tests were made immediately upon mixed castor bean preparation and water for control. Phenyl acetate and glyceryl triacetate, 3.4 milli-equivalents of each, were used. The mixtures contained 0.1 gm. of castor bean preparation and 15 cc. of water. Toluene was present throughout. The usual precautions were

[TABLE V.

Lipase Actions of Castor Bean Preparation at Different Temperatures after Standing with Water.

Ester.	Temperature of enzyme tests.	Mixed, tested immediately.	Mixed, stood 24 hrs. at 5–7°, then tested.	Mixed, stood 24 hrs. at 37°, then tested.	Mixed, tested immediately.	Mixed, stood 48 hrs. at 5–7°, then tested.	Mixed, stood 48 hrs. at 37°, then tested.
	°C.						
PhOAc	5–7	3.67	2.52	1.17	3.79	2.28	0.50
"	37	2.15	2.02	1.49	1.96	1.95	1.31
Gl(OAc)$_3$	5–7	0.31	0.28	0.17	0.31	0.20	0.12
"	37	1.04	0.93	0.81	0.96	0.99	0.63

taken, and the enzyme actions given as tenths of milli-equivalents of ester hydrolyzed under the given conditions. The results of Experiments 67 and 68 are combined and shown in Table V.

The mixtures, tested immediately upon mixing, showed considerably greater actions on phenyl acetate at the lower temperature, and greater on glyceryl triacetate at the higher. Standing at the lower temperature for 24 or 48 hours and then tested at the same temperature, the actions on phenyl acetate were about two-thirds as great as when tested immediately. Standing at the lower temperature and tested at the higher, the actions on phenyl acetate were practically the same as when tested imme-

diately at the higher temperature. The actions after standing at the lower temperature 24 or 48 hours and then tested at the two temperatures with phenyl acetate, showed somewhat greater actions at the lower temperature, but not as much greater as when tested without standing. Standing at 37°, more inactivation resulted than at 5–7°. Here, after 24 or 48 hours at 37°, the actions on phenyl acetate were greater at the higher temperatures, the difference becoming greater the longer the time of standing.

The actions on glyceryl triacetate were larger in every case at the higher temperature. Standing at 5–7° and then testing, very little loss in action was observed; standing at 37° and then testing, the actions were found to have decreased considerably, especially after standing 48 hours.

DISCUSSION.

Some definite conclusions may be drawn from the last experiments shown. Part of the enzyme of the castor bean acting upon the phenyl acetate is unstable on standing in water solution or mixture. This unstable enzyme is the reason for greater actions being found with phenyl acetate at the lower temperature. The process of incubation at the higher temperature in testing the enzyme action is sufficient to inactivate part of the enzyme. These facts are confirmed by the results of the actions after different periods of time given earlier. Greater actions were found at the higher temperature for periods less than 6 to 8 hours; after that the actions were greater at the lower temperature. It was there shown also that part of the enzyme was comparatively heat-stable (at 55°).

The temperature effects observed with phenyl acetate were not found with glyceryl triacetate. Here, as well as with the other esters tested and for which the results were presented, greater actions were found at the higher temperature. Also, the heat lability of this enzyme action was not nearly as large as that of the phenyl acetate-enzyme action. It seems difficult to draw any other conclusion but that there are at least two enzymes acting here. Whether two or more enzymes are assumed to be acting, or whether the same active enzyme grouping is assumed to be present in different molecules which influence the stability of the grouping, amounts essentially to the same thing in the final

analysis. A number of additional experiments was performed for which the results are not given here. With phenyl acetate as the substrate, the great sensitivity of the enzyme was repeatedly brought out. Irregular or contradictory results were obtained at times, but these, on closer study, were found in almost every case to be due to changed conditions. For instance, making up a mixture or extract, allowing it to stand 3 or 4 days in the ice box before testing, might show results quite different from those obtained by testing the freshly prepared mixture or solution.

The conclusions derived from the more intensive study of the castor bean may be carried over to explain the results obtained with eels and trout. With eels, the enzyme relations appeared to be essentially the same as with castor beans, greater action on phenyl acetate under the given conditions at the lower temperature, smaller actions on the other esters studied. With trout, the relations appear to be more complicated. The age of the trout appeared to play a part. Other esters, rather than phenyl acetate, showed greater actions at the lower temperature, especially benzyl acetate. Possibly a greater number of enzymes are present in trout, especially at certain ages; in any event, the existence of different enzymes must be assumed.

The explanation of the greater actions at the lower temperatures, where these were found to occur, is evidently the one indicated at the beginning of this paper. Inactivation by heat of the enzyme during the course of the enzyme test as balanced against increase in rate of reaction due to rise in temperature, results, under certain conditions, in the larger actions at the lower temperatures. At the same time, another possible factor must be mentioned. The substrate itself may inactivate the enzyme[3] and the rate of this inactivation with increase in temperature may play a part in producing apparently abnormal results. This influence is possibly present with phenyl acetate in the experiments described but the results are not sufficiently clear-cut to prove it.

The effect of observations of the kind described here in connection with studies of the kinetics of the actions are sufficiently obvious and need not be discussed further. Other enzyme actions may show relations under certain conditions similar to those de-

[3] Falk, K. G., *J. Am. Chem. Soc.*, 1913, xxxv, 616.

scribed here with lipase actions and their study therefore be correspondingly complicated.

Thanks are due Mr. Edmond White for his assistance in carrying out the experimental work described in this paper.

Finally, the writers wish to take this opportunity to thank Dr. C. B. Davenport for placing the facilities of the laboratories of the Station for Experimental Evolution of the Carnegie Institution at their disposal, as well as for his helpful aid and advice in this investigation.

SUMMARY.

Based upon studies described elsewhere, the ester-hydrolyzing actions on a number of esters, by eels, trout, and castor beans, both solid and in the form of extract, were studied at temperatures between 5° and 55° in different experiments.

The actions, under definite conditions of testing, were found in a number of cases to be considerably greater at the low temperatures. Thus, with eels, the action was greater at 15° than at 37° on phenyl acetate for a 22 hour enzyme test, but the actions on all the other esters studied were greater at the higher temperature; with trout, the actions on benzyl acetate and some of the other acetates were greater similarly at the lower temperature, but here the age of the trout played a part; with castor beans, the actions were larger on phenyl acetate at the lower temperature if the actions were tested for 8 hours or longer. Part of the lipase of the castor bean showed a marked stability at 55°.

The explanation of the greater enzyme actions at the lower temperature was shown to be the result of two opposing actions; increase in enzyme action due to rise in temperature, and inactivation of enzyme by heat in the course of the experimental tests.

The bearing of these results on enzyme tests in general and on the possible structure of these enzymes is briefly discussed.

THE FORMS OF LACTIC ACID PRODUCED BY PURE AND MIXED CULTURES OF BACTERIA.*

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A relation between stereoisomerism and biological activity extends through the chemistry of the sugars, peptides, amino acids, and many other organic compounds. The production of the two enantiomorphs of lactic acid by biological agents is one example of this relationship. In the animal organism it is generally assumed that sarco- or *d*-lactic acid is the exclusive form produced while in the field of microbiology both the *d* and the *l* forms have frequently been found. It has been reported that some bacteria, like the animal cell, produce only one type of acid whether it be the *d* or the *l* form.

A critical examination of the published data leads to the conclusion that the exclusive production of but one form of lactic acid by either an animal or plant cell rests on somewhat doubtful evidence. The water of crystallization and the specific rotation of the zinc lactates are the principal data which bear upon this question. Usually only the first crop of zinc lactate has been examined for the water of crystallization and in some cases the zinc salts have been recrystallized before examination. Both of these procedures would fail to show the presence of small quantities of either enantiomorph in the presence of large quantities of the other. The values given for the specific rotation of the zinc lactates of plant or animal origin vary so greatly that if any conclusion is to be drawn it is that a mixture of the two forms was present rather than that a single enantiomorph was under examination.

* Published with the permission of the Director of the Wisconsin Agricultural Experiment Station, Madison.

Embden and his associates (1–5) have done the most extensive work on the type of lactic acid produced by the animal cell and while they found *d*-lactic acid in most cases, under certain conditions they found an excess of *l*-acid. There can be no doubt that the animal cell has the ability to produce both forms. Changes in the media have been reported by Kayser (6), Pere (7), and Pottevin (8) as resulting in a change in the form of lactic acid produced by bacteria. It is not improbable that such bacteria always produce both forms but under ordinary conditions the product is mainly one type. In this paper it will be shown that certain of the lactic acid bacteria when grown in the presence of non-lactic acid bacteria will produce both kinds of acid. It will also be shown that certain microorganisms which appear to produce equal quantities of both forms, in reality produce an excess of one enantiomorph.

EXPERIMENTAL.

Cultures and Media.—The organisms used were pure cultures obtained from various sources, some of them producing mainly lactic acid and others producing a variety of products. Culture R is a strain of *Streptococcus lactis* (9), Culture 60 an unknown streptococcus, Cultures A, C, and D strains of *Lactobacillus leichmanni* (10), Cultures 41–11 and 26 mannitol-forming lactic acid bacteria (11), and Culture 105 a strain of the acetone butyl alcohol-forming organism.

Yeast water containing 3 per cent of the various sugars was used as culture media. After inoculation, an excess of $CaCO_3$ was added and the culture incubated at 28 or 37°C. In certain experiments the yeast water was replaced by a 6 per cent corn mash medium. When 10 days old a sample was withdrawn and the percentage of unfermented sugar determined by the Shaffer and Hartmann (12) method.

Chemical Methods.—The fermented culture was acidified with sulfuric acid, steam-distilled to remove volatile acids, and the residue extracted in a Kutscher and Steudel apparatus with ether. After evaporating the ether, an aliquot was titrated to determine the quantity of extracted acid. The remainder was decolorized with norite and boiled with zinc carbonate to form the zinc salts. The solution was fractionally crystallized at 37°C., the fractions

dried for a few hours in the air and then for about 24 hours over calcium chloride. The water of crystallization was determined by heating at 104–106°C. for 3½ to 4 hours. That the temperatures used in this procedure are not high enough to change the form of lactic acid produced was shown by subjecting a known active acid to these methods and redetermining the acid. If the acid were changed, this would undoubtedly occur during steam distillation or in boiling with zinc carbonate. Therefore this test solution was steam-distilled for a much longer period of time than is ordinarily used. Of the 22.179 gm. of zinc salt used, 92.5 per cent was recovered. The water of crystallization was 12.92 per cent, showing that the form of the lactate remained unchanged by the analytical procedure.

Determination of Optical Rotation.—The optical rotation of the salts was determined in some cases to check the water of crystallization data and to decide if the active acid was dextro- or levorotatory. In 25 cc. of distilled water were dissolved 1.0305 gm. of the anhydrous salt and the rotations produced in a 2 dm. tube were read upon the Ventzke scale and converted to the corresponding specific rotation. Hoppe-Seyler and Araki (13) with comparable concentrations and temperatures, found active zinc lactate to have a specific rotation of ± 7.52. They also showed that the zinc salt had the opposite rotation to the free acid. Irvine (14) and Purdie (15) have obtained rotations ranging from ± 7.74 to ± 7.85 under the same conditions. While most investigators report figures close to this value, others give results ranging from ± 6.7 to ± 9.9 (1–5). It would seem as if such divergent figures must be due to errors in analytical procedure.

Form of Lactic Acid Produced by Pure Cultures of Various Strains of Lactic Acid Bacteria.—In this experiment three kinds of bacteria were used; a strain which produces *d*-lactic acid, one which produces *l*-lactic acid, and a third which produces both types. The kind of sugar fermented was also varied. In all cases from 70 to 90 per cent of the sugar was destroyed and from 70 to 90 per cent of the extracted acid was recovered as the zinc salts. The data are given in Table I. The water of crystallization and the specific rotation show that Culture R produced mainly dextro acid and but little levo, while Culture A did just the reverse. Cultures 41–11 and 26 produced large quantities of both forms but a con-

siderable excess of the *d*-acid. Culture 60 formed more levo than dextro acid. In interpreting the data it must be borne in

TABLE I.

Form of Lactic Acid Produced by Pure Cultures of Various Bacteria.

Culture No.	Sugar.	Zinc lactate.				Rotation of fractions.
		Crop No.	Weight.	Water of crystallization.		
				Fractions.	Average.	
			gm.	*per cent*	*per cent**	$[\alpha]_D^{20}$
R	Glucose	1	7.187	13.2		−8.11
	15 gm.	2	2.122	13.0		
		3	1.425	13.2	13.2	
R	Lactose	1	14.485	12.8		
	30 gm.	2	4.020	13.3		
		3	1.441	13.0	12.9	
A	Glucose	1	12.519	12.9		
	30 gm.	2	6.277	12.9		+7.39
		3	1.326	13.2	13.0	
C	Glucose	1	3.893	13.0		
	6 gm.	2	1.366	13.9	13.4	+6.93
41-11	Glucose	1	5.746	18.1		
	30 gm.	2	3.458	17.9		
		3	2.533	13.3	17.0	−7.15
41-11†	Glucose	1	8.805	18.2		
	15 gm.	2	0.996	17.3	18.1	
41-11	Fructose	1	4.539	18.2		
	21 gm.	2	1.917	17.4	17.9	
41-11	Xylose	1	4.555	18.1		
	9 gm.	2	0.727	17.7		
		3	0.750	15.2	17.7	
26	Glucose	1	2.455	18.2		
	15 gm.	2	2.537	16.5		−1.52
		3	1.518	13.3	16.6	
60	Glucose	1	0.891	18.0		
	6 gm.	2	0.764	14.9	16.5	+1.13

* Inactive zinc lactate contains 18.2 per cent and active zinc lactate 12.9 per cent water of crystallization.

† Fermented at 37°C.

mind that a mixture of the two forms of lactic acid results in the formation of inactive zinc lactate and that the active zinc salt has the opposite rotation of the free acid.

Fractional crystallization of the zinc salts is necessary to show clearly the presence of an excess of one form of acid. It is in the second and third crops that the excess of one form is made evident. This is especially noticeable with Cultures 41–11, 26, and 60. The first crop is all inactive zinc lactate in every case, the second crop shows distinctly the presence of some active salt, and the third crop is almost entirely the active compound. The specific rotation shows this to be *l*-zinc lactate.

Tate (16) reported that the kind of sugar fermented had a marked effect on the type of acid produced but with our cultures no particular effect was observed. One glucose culture was incubated at 37°C. and showed a somewhat greater production of inactive acid at the higher temperature. While the data in Table I are not conclusive they are strongly in support of the view that *perhaps all lactic acid bacteria produce both dextro and levo lactic acid.* In some cases and under certain conditions one form may be produced in minimal quantities but it is probably never entirely absent.

Temperature As a Determining Factor in the Development of Mixed Cultures.—In many natural fermentations such as milk, silage, and sauerkraut, temperature is probably the most important factor in determining the initial fermentation. If the temperature of the medium is particularly favorable for the growth of certain bacteria these strains will develop more rapidly than other types and thus to a greater or less extent dominate the fermentation. If the microorganism under study produces a characteristic fermentation product such as alcohol or lactic acid this product may be of value as an index of growth. Numerous investigations (17–24) have been made showing that changes in temperature have a decided effect on the kind of acid produced. Since Cultures R and A produce opposite forms of lactic acid (9,10) and No. 41–11 produces ethyl alcohol from the aldohexoses (25) it was felt that a study of the products formed in mixed culture would throw some light on the question as to which type of organism predominated in the fermentation. The results are given in Table II. At 15°C. pure dextro acid is formed, showing that the acid is produced by Culture R. As the temperature is raised, an increasing amount of levo acid is produced, until at 37 and 45°C. a pure levo acid results.

Although microscopical examination showed the presence of both organisms in all of the cultures, the growth of Culture A at low temperatures and of Culture R at high temperatures is

TABLE II.

Effect of Temperature on the Forms of Lactic Acid Produced by Mixed Cultures.

Culture No. and inoculum.	Sugar.	Temperature.	Zinc lactate.			Rotation of fractions.	Percentage of sugar converted into ethyl alcohol.
			Crop No.	Weight.	Water of crystallization.		
		°C.		gm.	per cent	$[\alpha]_D^{25}$	
Series I. Cultures R and A.							
1:10	Glucose.	15	All.*	11.203	13.3	−7.14	
10:1	"	15	"	9.161	13.1		
1:1	"	22	"	20.543	15.6	−3.75	
1:10	"	28	"	15.247	17.7		
10:1	"	28	"	14.708	18.1		
1:10	"	37	"	19.292	12.9	+7.57	
10:1	"	37	"	15.613	13.4	+7.27	
1:1	"	45	"	21.653	13.0	+7.44	
Series II. Cultures R and 41–11.							
1:1	Glucose 15 gm.	28	1	3.430	17.9		6.9
			2	3.454	13.3	−7.57	
			3	0.890	13.9	−6.76	
1:1	Sucrose 15 gm.	28	1–3	8.743	18.1		15.5
1:1	Lactose 15 gm.	28	1–2	3.560	17.3		11.6
			3	1.529	13.9	−5.33	
1:1	Milk 1300 cc.	28	1	2.193	16.7		
			2	2.398	13.6		
			3	1.312	12.6		

* From 80 to 95 per cent of the extracted acid was recovered as the zinc salt.

comparatively slow. A number of workers (17–20), experimenting with spontaneous milk fermentations, have found that at low temperatures there is always a tendency for the organisms producing dextrorotatory acid to grow, and as the temperature is

increased, the organisms producing the inactive or levo form are accelerated. It is to be noted that when the ratio between the two organisms is large, the same result is obtained as when the ratio is 1. Therefore the form of acid produced is determined by the temperature and not by the amount of inoculum.

In the next series Cultures R and 41–11 were used and the kind of sugar in the media was varied. Culture R produces dextro acid, while Culture 41–11 produces inactive acid, ethyl alcohol, and other products. Culture 41–11 grows but slowly on lactose and not at all on milk, while Culture R is a vigorous fermenter of such solutions. In spite of this, large quantities of inactive acid as well as alcohol were produced. These are both indicative of the growth of Culture 41–11. From the amount of alcohol produced from glucose, sucrose, or lactose it was estimated that from one-third to two-thirds of the sugar was fermented by Culture 41–11. The percentage of inactive acid produced indicates even a more extensive growth of Culture 41–11. The discrepancy between the alcohol and lactic acid determinations may be explained, however, by assuming that the presence of Culture R causes Culture 41–11 to produce more levo acid than is its wont when growing by itself. It is possible that the presence of a considerable quantity of one form of acid may stimulate the microorganism to produce the opposite form in an attempt to keep a balance between the two enantiomorphs.

Form of Acid Produced in the Presence of a Non-Lactic Microorganism.—The effect of mixing a non-lactic culture, the acetone butyl alcohol organism (Culture 105), with a lactic culture, *Lactobacillus leichmanni* (Culture A), was studied in the next experiment, Table III. The quantity of inoculum was also varied to show the effect of this factor on the growth of the two organisms. The growth of Culture A in an unchanged corn mash (6 per cent) medium is slow, but Culture 105 grows well with the production of the solvents, acetone, ethyl alcohol, and butyl alcohol, and small amounts of leucic acid. The percentage of solvents, Column 5, is indicative of the action of Culture 105 and the percentage of lactic acid, Column 6, is an index of the growth of Culture A. It is to be noted that the amounts, 0.02 to 1.27 per cent of solvents and 0.01 to 0.40 per cent of lactic acid, are proportional to the amount of inoculum. In the flasks inoculated

with a large quantity of Culture 105 and a small quantity of Culture A the former microorganism dominated the fermentation and produced a large percentage of solvents. At the other end of the series the conditions were reversed. Most of the mash remained unfermented and a high percentage of lactic acid was produced. Culture A produces a levo form on glucose yeast water almost exclusively, but in the presence of Culture 105 it

TABLE III.

Effect of the Associated Growth of Non-Lactic and Lactic Acid-Producing Bacteria.

Flask No.	Volume of media.	Inoculum.		Solvents.	Non-volatile acid as lactic.	Zinc lactate.				Rotation of fractions.
		Culture 105.	Culture A.			Crop No.	Weight.	Water of crystallization.		
								Fractions.	Average.	
	cc.	*cc.*	*cc.*	*per cent*	*per cent*		*gm.*	*per cent*	*per cent*	$[\alpha]_D^{20}$
1	1800	20	0	1.27	0.01	All.	None.			
2	5400	60	3	1.12	0.11	1	6.643	17.7		
						2	2.400	14.7	16.9	+6.26
3	1300	25	1.2	1.07	0.18	1	3.018	16.6		
						2	0.380	14.4	16.3	+7.44
4	1640	20	5	1.05	0.18	1	1.060	17.6		
						2	2.624	14.1	15.1	+7.14
5	1550	20	10	0.02	0.40	1	5.281	15.6		
						2	1.707	15.0	15.5	+6.89
6*	500	0	5			All.	None.			
7*	500	0	5			"	1.487	13.1		
8†	1000	20	20			"	10.580	13.1		+7.73

* Flask 6 was digested with diastase and Flask 7 with pancreatin before sterilization and inoculation.

† 18 hours after inoculation with Culture 105 the flask was sterilized and inoculated with Culture A.

produces large quantities of dextro as well as levo acid. Even when Culture 105 appeared to make but little growth as in the fifth analysis, its presence was a powerful influence in stimulating the production of *d*-lactic acid by Culture A. By itself (see Table I) it produces scarcely any of the *d*-form but when mixed with Culture 105 from 25 to 40 per cent of the total acid produced consists of the *d*-enantiomorph.

That it is not the corn mash media nor the chemical products of the acetone butyl alcohol organism which brings out the production of *d*-lactic acid by *Lactobacillus leichmanni* is shown by the results obtained in two other fermentations. In Flasks 6 and 7 the mash was digested with diastase and pancreatin respectively to improve the quality of the media. While there was plenty of reducing sugar in both flasks Culture A grew only in the mash digested with pancreatin. Apparently the undigested proteins of corn mash are unavailable to Culture A. This assumption was shown to be correct by adding 1 per cent of peptone to the mash after digesting with diastase. In this medium it grew well and the acidity rose to 13.2 cc. of 0.1 N acid per 10 cc. of mash as compared to 4.2 cc. in a medium to which no peptone was added. The lactic acid produced by Culture A in the mash digested with pancreatin was determined and found to be almost entirely *l*-acid. This is the same as that produced in glucose yeast water. The kind of carbohydrate or protein can therefore not be the determining factor in the production of *d*-lactic acid.

That the acetone, butyl alcohol and other fermentation products of Culture 105 are not responsible for the appearance of *d*-lactic acid was demonstrated by the data obtained from Flask 7. The mash was fermented by Culture 105 for 18 hours and a sample withdrawn for analysis. At this time a considerable quantity of reducing sugar, soluble protein, volatile acids, and some solvents had been formed by the butyl alcohol organism. No zinc lactate, however, could be obtained from the partly fermented material. The remainder was sterilized, an excess of sterilized $CaCO_3$ added, and inoculated with Culture A. The organism grew well and after 7 days was analyzed for lactic acid. More than 10 gm. of zinc lactate were obtained and the water of crystallization and specific rotation show that this was almost entirely *l*-lactic acid. The growth of Culture A is not inhibited by the fermentation products of Culture 105 nor are these products capable of causing the production of *d*-lactic acid by Culture A. It appears that the living cell or some heat-labile substance produced by the cell must be present to produce this effect.

Form of Lactic Acid Produced in Natural Fermentations.—The lactic acids prepared by Merck and by Dupont were found to be inactive with some excess of the levorotatory modification (Table

IV). These results are the same as those obtained by Phelps and Palmer (26). Without doubt, all commercial lactic acids would be found to contain mostly inactive acid since they are produced by mixed cultures of organisms. A sample of acidophilus milk was found to contain a slight excess of levorotatory acid. Since the milk was not sterilized before inoculation, the results obtained are comparable to those found with naturally soured milks.

The acid produced in sauerkraut was also found to contain a slight excess of the levorotatory modification. An attempt was

TABLE IV.

Form of Lactic Acid Produced in Natural Fermentations.

Source.	Crop No.	Zinc lactate.	Water of crystallization.		Rotation of total fractions.
			Fractions.	Average.	
		gm.	*per cent*	*per cent*	$[\alpha]_D^{25}$
Dupont.	1	16.6200	18.05		
	2	2.6654	17.75		
	3	1.3190	17.73		
	4	9.0124	13.53	16.60	+2.20
Merck.	1	8.1070	17.96		
	2	3.6710	15.90		
	3	5.2572	14.80	16.54	+2.27
Acidophilus milk.	1	2.9628	18.09		
	2	2.1977	14.92	16.74	+6.72*
Kraut.	1	11.9738	18.15		
	2	2.4206	15.32	17.53	+1.85
Kraut inoculated with Culture R.	1	4.1679	18.04		
	2	1.4049	13.72	16.94	−1.18

* Rotation of Crop 2.

made to change the form of acid in kraut by adding a large inoculum of Culture R, equivalent to 2 per cent by weight of the cut cabbage. A slight excess of the dextrorotatory acid was produced, showing that the acid in a natural fermentation can be changed by inoculation. As the temperature at which sauerkraut is made, 17–22°C., has already been shown to favor the growth of Culture R, the results obtained are those to be expected.

Solubility and Fractional Crystallization of the Zinc Lactates.—Attention has already been called to the value of fractional crystallization of the zinc lactates as a means of demonstrating the pres-

ence of small quantities of one form of lactic acid in the presence of large quantities of the other. When the work reported in this paper was partly completed, it was decided to determine the solubility of the zinc lactates and the effect of mixtures of the inactive and the dextro salts on the solubility of each component.

The solubility of the three forms of zinc lactate was determined by Wislecenus (27) and by Purdie (15) as 1 part in 57 for inactive zinc lactate and 1 part in 17 for the active salts. The results

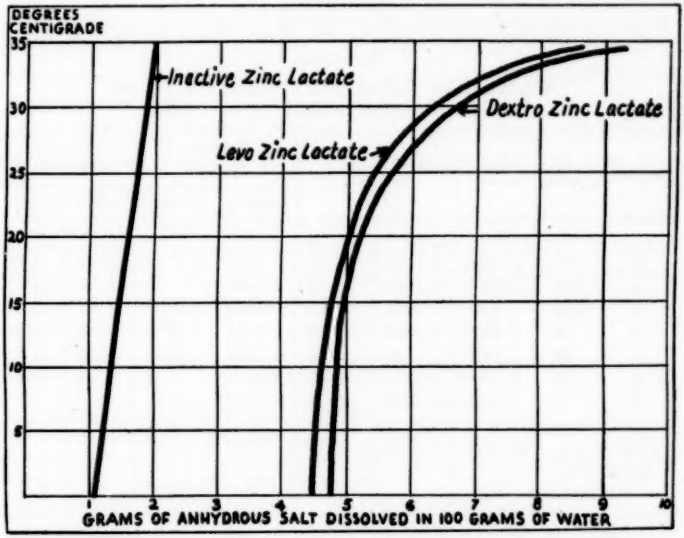

CHART I. Curves showing the solubility of the three isomeric forms of zinc lactate.

shown in the graph (Chart I) are in agreement with these investigators, but above 25° the solubility of the active salts increases decidedly with an increase in temperature. Because of this increase in solubility, a much better separation of the active from the inactive salt can be made at 35°C. The active salts, especially the dextro, have a decided tendency to form supersaturated solution leading to difficulties in solubility determinations. In this work the salts were allowed to crystallize from 24 to 48 hours with frequent shaking before samples were removed.

The order of crystallization of the inactive and dextro salts at 35°C. was next determined. The temperature, the change of solubility due to the change of concentration of the two salts, and the fact that zinc lactates readily form supersaturated solutions (28) all have a bearing upon this matter. 5 gm., each of anhydrous inactive and active zinc lactate, were dissolved in 360 cc. of water. This was evaporated and seven crops taken. The results in Table V show that 84 per cent of the inactive salt is crystal-

TABLE V.

Order of Crystallizations of Inactive and Active Zinc Lactates from Mixed Solutions.

Weight of Zn $(C_3H_5O_3)_2 \cdot 3H_2O$ taken = 6.112 gm.
" " " $(C_3H_5O_3)_2 \cdot 2H_2O$ " = 5.741 "

Crop No.	Volume of water.	Zinc lactate.	Loss of water at 105°C.		Zinc lactate in each crop.		Zinc lactate in solution.	
					Inactive.	Active.	Inactive.	Active.
	cc.	*gm.*	*gm.*	*per cent*	*gm.*	*gm.*	*per cent*	*per cent*
1	251	0.8484	0.1530	18.03	0.8315	0.0169	2.02	2.25
2	229	1.0294	0.1874	18.19	1.0294		1.76	2.46
3	166	1.6148	0.2894	17.90	1.5341	0.0807	1.57	3.35
4	84	2.3905	0.3896	16.29	1.5539	0.8366	1.14	5.60
5	49	2.5810	0.3588	13.90	0.5007	2.0803	0.94	5.37
6	24	1.3281	0.1854	13.88	0.2523	1.0758	0.86	6.47
7	0	1.1152	0.1489	13.35	0.1005	1.0147		
Total.....		10.9074	1.7125	15.69*	5.8024	5.1050		
Recovery, *per cent*....		92			95	89		

* Calculated from the totals in Columns 3 and 4. Theory for equal quantities of active and inactive zinc lactate, 15.55 per cent.

lized out in the first four crops, while 89 per cent of the active salt appears in the last four crops. This is a fairly good separation, but apparently it is impossible to make a total separation of the inactive salt from the active modification. The figures in the last two columns show the effect of one salt on the solubility of the other. According to Chart I the solubility of inactive zinc lactate at 35°C. is 2 per cent and of levo zinc lactate is about 9 per cent. When both salts are present in the same solution these figures are much reduced. For example after the removal of

Crop 4, the saturated solution contains but 1.14 per cent inactive and 5.60 per cent levo zinc lactate. After the sixth crop these figures have decreased to 0.86 per cent and increased to 6.47 per cent respectively. From these data it can be seen that a clean cut separation of the two types would involve an extended series of crystallizations.

SUMMARY.

In pure cultures bacteria may produce dextro or levo lactic acid almost exclusively. When grown in mixed cultures with an organism which does not produce lactic acid large quantities of the opposite enantiomorph may be produced. It is possible that both forms of acid are produced by all lactic acid bacteria. In some cases the production of the two forms is nearly equal while in other cases the point of equilibrium is near 100 per cent production of one of the enantiomorphs.

The solubility of the three zinc lactates has been determined. In a mixture of two zinc lactates the solubility of each component is influenced by the quantity which is present of the other component.

A separation of inactive and active zinc lactates is best effected at or above 35°C.

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DOES THE ADDITION OF SODIUM CHLORIDE INCREASE THE VALUE OF A CORN RATION FOR GROWING ANIMALS?*

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The numerous investigations of McCollum and his associates concerned with the dietary deficiencies of natural foods have contributed several interesting and valuable view-points to the science of nutrition, and have stimulated a large amount of research of a similar character. Among these new view-points, the conclusion that the dietary value of a food material, whether animal or vegetable, is related to its function in the organism from which it was derived, and, conversely, that anatomical parts of like function are similar in nutritive value, may be mentioned. Unfortunately, the biological method of analysis of foods used throughout McCollum's investigation is not capable of yielding results susceptible of only one interpretation, mainly because the only factors under control have been the composition of the rations compared and the choice of animals used. No attempt has been made to equalize food intake among groups of animals compared, although the amount of food consumed by an animal, as well as its composition, must affect the rate of growth and the reproductive performance. Hence, although the results of these and similar investigations have been proven to be largely correct, in so far as they have been checked by other methods, they have not been found to be wholly so. The generalizations deduced from them have gained currency more because of their simplicity and apparent rationality than because of their infallibility.

* The experimental data presented in this report were taken from the thesis of Mr. Carman submitted in partial fulfillment of the requirements for the degree of Master of Science in Animal Husbandry in the Graduate School of the University of Illinois, 1925.

An instance illustrating the success as well as the faults of the biological method of the analysis of food materials relates to the inorganic moiety of seeds. McCollum's investigations, in conjunction with other investigations in which different experimental methods were used, have established the fact that seeds as a class are deficient in mineral matter for growing animals. With regard to the particular inorganic elements occurring in seeds in deficient amounts, however, the evidence is not so convincing. In a publication dated 1917, McCollum, Simmonds, and Pitz (1) conclude that seeds as a class are deficient in sodium, calcium, and chlorine, merely on the basis of chemical analyses. In a later publication of the same year, McCollum and Simmonds (2) endeavor to determine the exact nature of the mineral deficiencies of a mixture of maize and navy beans. Since this is the only investigation of this nature referring to seeds of which the authors are aware, a detailed consideration of the results seems warranted. The charts bearing directly upon this question may be described as follows:

Chart 17, Lot 814. On a ration of corn 74.1 per cent, beans 19.0, $CaCO_3$ 0.9, NaCl 1.0, and butter fat 5.0, normal growth and good reproduction were secured, though only 15 of the 42 young produced were reared.

Chart 18, Lot 810. A ration containing corn 78.1, beans 19.0, potassium citrate 1.9, and NaCl 1.0 produced practically no growth.

Chart 18, Lot 809. A ration of corn 78.2, beans 19.0, $CaCO_3$ 0.9, and potassium citrate 1.9 produced only moderate but nevertheless continuous growth. A substitution of 1.0 per cent NaCl for the potassium citrate had no distinct effect upon the growth curves. However, the initiation of pregnancy in the only female of the group was coincident with this change.

Chart 19, Lot 813. A ration of corn 73.2, beans 19.0, potassium citrate 1.9, NaCl 1.0, and butter fat 5.0 produced a moderate but sustained rate of growth for over 6 months. An addition of $CaCO_3$ at this time had no appreciable effect on the growth curves.

Chart 20, Lot 812. A ration of corn 73.2, beans 19.0, $CaCO_3$ 0.9, potassium citrate 1.9, and butter fat 5.0 supported a slow but sustained growth for about 5 months, at which time an addition of NaCl, while producing no evident effect on growth, was coincident with the initiation of a single pregnancy in one female and of three successive pregnancies with the other.

Chart 21, Lot 921. A ration of corn 74.0, beans 19.0, NaCl 2.0, and butter fat 5.0 supported a moderate and continuous growth, not appreciably bettered by the addition, after 3 months of feeding, of 1.8 per cent of $CaCO_3$.

Chart 22, Lot 916. A ration of corn 79.2, beans 19.0, and $CaCO_3$ 1.8 gave practically no growth, nor did the addition of $CaCl_2$ improve matters.

Chart 23, Lot 979. Excellent growth was obtained for 2½ months on a ration of corn 73.7, beans 19.0, $CaCO_3$ 0.9, $NaHCO_3$ 1.4, and butter fat 5.0.

The evidence in these charts with reference to the supplementing effect of NaCl is singularly divided, while even the incorporation of a calcium salt in the ration did not produce the distinct beneficial effect to be expected from other experiments demonstrating its marked deficiency in seeds. It is unfortunate that the experiment on Lot 979, Chart 23, was not continued longer than 2½ months.

In all probability the discordant and, at times, unexpected results of these feeding experiments could be explained if food intake records had been reported, or could have been obviated entirely if the food intakes of otherwise comparable groups had been equalized. In experiments dealing with a material of such a pronounced condimental effect as common salt, some measure of dietary control is necessary to the proper interpretation of changes in rate of growth coincident with its addition to the ration.

The results of the experiment above reviewed are offered as complete proof "that sodium and calcium are the only inorganic elements which need to be added to make good the mineral deficiencies of maize and beans," but are cited in a later publication (3) as demonstrating, in conjunction with an experiment concerned with the production of rickets on low phosphorus diets (4), "that the deficiency in mineral elements in wheat and other seeds is limited to four elements, calcium, phosphorus, sodium, and chlorine." It may be added that oats, soy beans, cow-peas, peanuts, flaxseed, and cottonseed are known (5) to contain several times as much sodium as either corn or navy beans.

With reference to the concentration of sodium and chlorine in the diet requisite for normal growth in the rat the results of Osborne and Mendel (6) are not readily reconciled with the conclusions of McCollum and Simmonds. On rations containing 0.035 per cent of sodium or of chlorine, rats were able to complete their growth at a vigorous rate, in one case at a rate considerably greater than "normal." This concentration of sodium is about the same as that in corn, while the concentration of chlorine in corn is considerably higher than 0.035 per cent according to published analyses.

The divergence between the conclusions of McCollum and the data of Osborne and Mendel with reference to the minimum concentration of sodium and chlorine in the diet requisite for normal growth, rather than the practical aspects of the question, led to the undertaking of the experiment reported in this paper. Since corn (maize) contains concentrations of sodium and of chlorine close to the concentrations of these elements in the rations used in the experiments of McCollum, as well as of Osborne and Mendel, the ration used in this experiment was made up largely of this cereal. By feeding to growing animals a ration largely composed of corn and properly supplemented with respect to protein, fat-soluble vitamins, and calcium, with and without an addition of NaCl, it was hoped to determine whether a concentration of sodium and chlorine approximating 0.04 per cent is adequate for maximum growth. The food intakes of animals whose performance it was desired to compare with reference to the effect of the NaCl supplement, were equalized in this experiment, either absolutely or in proportion to their maintenance requirements of energy.

EXPERIMENTAL.

The experiments consisted of growth observations and balance studies, the same animals being used for both. Sixteen albino rats and eight chicks were used as experimental subjects.

The rats—5 weeks old at the beginning of the experiment—were divided into eight pairs, one rat of each pair serving as a control. The two rats in each pair were litter mates of the same sex and weight, so that at the beginning of the experiment, the animals to be compared were as nearly alike as possible. One rat of each pair was fed a ration in which corn provided all the sodium and chlorine (Ration A), and the other animal of the pair was fed a ration containing additional sodium chloride (Ration B). The composition of the two rations was as follows:

Ration A.		*Ration B.*	
Ground corn	87	Ground corn	86
Casein	10	Casein	10
Cod liver oil	2	Cod liver oil	2
Calcium carbonate	1	Calcium carbonate	1
		Sodium chloride	1

Ration A contained on an average 0.047 per cent sodium, 0.041 per cent chlorine, and 2.58 per cent nitrogen, while Ration B contained an average of 0.481 per cent sodium, 0.724 per cent chlorine, and 2.57 per cent nitrogen.

The rats were kept in individual cylindrical wire cages (9 inches in diameter by 7 inches high) without bedding during the growth experiment of 12 weeks; records of food consumption were kept. The food intake with the first four pairs of rats was controlled so that the two animals of each pair received very nearly the same amounts of food each week. In the other four pairs the food intake of the animals was controlled approximately in proportion to the two-thirds power of the body weight, or roughly in proportion to body surface. The rat in each pair consuming the smaller amount of food determined the amount of food allowed its pair mate. The odd numbered rats received Ration A containing no added sodium chloride, while the even numbered rats received Ration B.

Food for the rats was weighed out once each week and kept in individual corked bottles. About equal parts of the amount

weighed out were fed daily moistened with distilled water, and the residues were collected, dried, and weighed back at the end of the week, when the average daily food consumption for the week was determined. The animals were provided with distilled water at all times.

At the end of the 12 weeks growth experiment on Rats 1 to 8, the animals were put in glass metabolism dishes for 7 days. During this period the food was weighed on an analytical balance and quantitative collections of urine and feces were made. The dishes used and the methods of taking collections were essentially the same as those described by Mitchell (7) in connection with the method used in this laboratory for nitrogen balance studies. The rats were supported on wire screens in the dishes, as described in a later paper (8).

Rats 9 to 16 were put in metabolism dishes after 4 weeks on the experimental diets, and, after the 7 day collection period, the growth experiment was continued to a total length of 12 weeks. Thus, balance data were secured on rats at about the ages of 9 and of 17 weeks.

The chickens used in the experiment were six young White Plymouth Rocks of the same age and two White Leghorns, also of the same age. They were divided into pairs, the two chicks in each pair being of the same sex and age, and approximately the same weight. They were kept in individual wire cages of the same size and pattern as those used for the rats. The growth experiment on the chicks was also of 12 weeks duration. The balance period on the chicks was begun at the end of the 2nd week of feeding, and lasted for 7 days.

The rations fed the chicks were of the same composition as those which the rats received, the odd numbered chick in each pair receiving Ration A and the even numbered chick receiving Ration B. With the chicks, as with the first four pairs of rats, the food intakes of the pair mates were kept the same from week to week.

During the growth experiments, all the experimental animals were weighed once a week. However, in view of the clear-cut results obtained, it has not been considered necessary to present the weekly statistics of weights and food consumption. A summary of these data is given in Table I, while the growth secured is graphically illustrated in Charts 1 to 3.

TABLE I.

Weight and Food Records of the Rats and Chicks during the Entire Growth Period.

Animal No.	Sex.	Ration.	Duration of experiment.	Initial weight.	Final weight.	Total gain.	Average daily feed.	Average daily gain.	Feed to produce 1 gm. of gain.
Rats.	**Pair mates received approximately the same amount of food.**								
			days	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
1	M.	A	82	58	179	121	11.56	1.48	7.8
2	"	B	82	59	225	166	11.42	2.02	5.6
3	"	A	82	54	172	118	11.27	1.44	7.9
4	"	B	82	55	242	187	11.44	2.28	5.0
5	F.	A	82	56	156	100	10.80	1.22	8.9
6	"	B	82	55	189	134	11.00	1.63	6.8
7	"	A	82	60	174	114	10.80	1.40	7.7
8	"	B	82	59	176	117	10.82	1.43	7.6
Rats.	**Pair mates received approximately the same amount of food per unit of body surface.**								
9	M.	A	84	68	176	108	10.78	1.37	7.9
10	"	B	84	68	265	197	11.81	2.35	5.0
11	"	A	84	59	165	106	10.11	1.35	7.5
12	"	B	84	59	261	202	11.88	2.40	5.0
13	"	A	84	60	143	83	9.49	1.31	7.2
14	"	B	84	60	264	204	11.84	2.43	4.9
15	F.	A	84	63	158	95	9.44	1.33	7.1
16	"	B	84	60	188	128	10.14	1.52	6.7
Chicks.	**Pair mates received approximately the same amount of food.**								
1	M.	A	84	164	413	249	21.39	2.96	7.2
2	"	B	84	161	603	442	21.25	5.26	4.0
3	F.	A	84	114	400	286	19.84	3.40	5.8
4	"	B	84	123	517	394	20.12	4.57	4.4
5	M.	A	84	184	405	221	21.12	2.63	8.0
6	"	B	84	180	557	377	21.30	4.49	4.7
7	F.	A	84	132	392	260	20.91	3.10	6.8
8	"	B	84	116	482	366	20.73	4.36	4.8

There were several differences in detail between the methods used for taking collections in this work and in nitrogen balance studies from this laboratory. The feces were collected in tubes

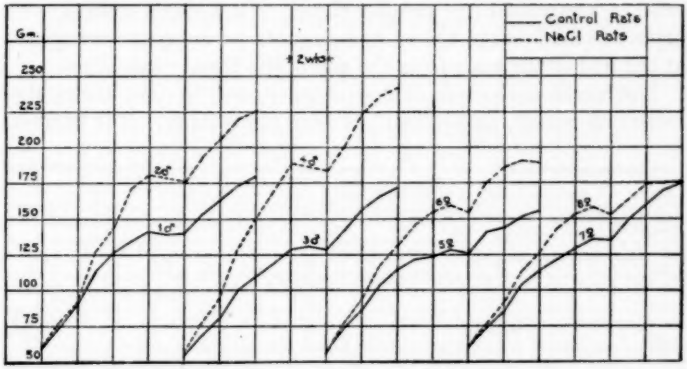

CHART 1. The growth of rats receiving a corn diet, supplemented with respect to protein, fat-soluble vitamins, and calcium, with and without the addition of NaCl. The food intake of both rats in each of the four pairs was kept the same throughout.

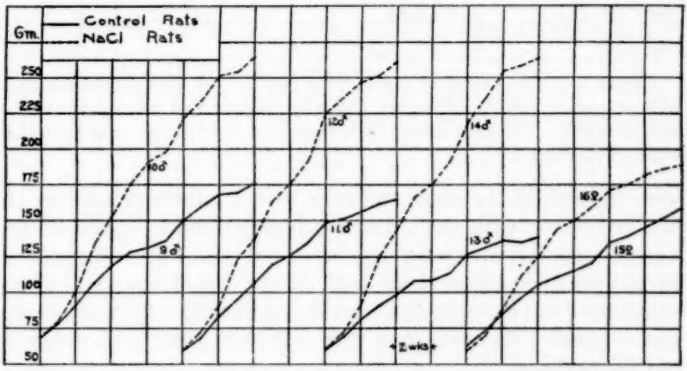

CHART 2. The growth of rats receiving a corn diet, supplemented with respect to protein, vitamins, and calcium, with and without the addition of NaCl. The food intake of both rats in each pair was kept the same per unit of surface area.

without alcohol, and were dried, weighed, and ground in a mortar at the end of the week. From this dried collection, portions were weighed out for analysis. The urine was washed out of the dishes with hot water acidulated with sulfuric acid (for the first eight rats) or acetic acid (for the last eight). Each day's collection of urine was made up to 250 cc. and aliquots for analysis were taken at the end of the week from the composite weekly collection.

The chicks were confined in wire cages over tin pans during the collection period, and collections were made daily. The excreta

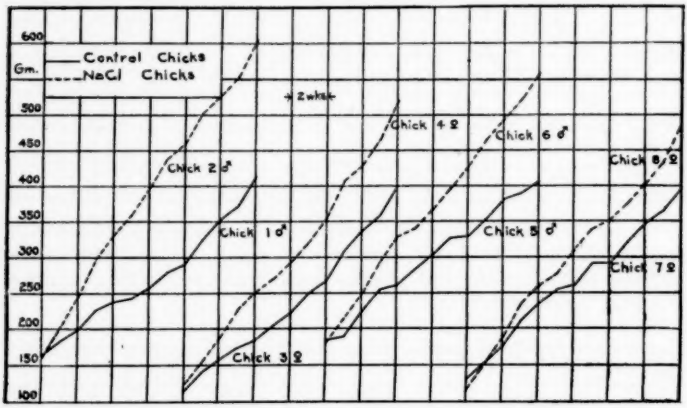

CHART 3. The growth of chicks receiving a corn diet, supplemented with respect to protein, vitamins, and calcium, with and without the addition of NaCl. The food intake of both chicks in each pair was kept the same throughout.

were washed off the cages and pans with a minimum amount of distilled water, with the aid of a rubber policeman. The whole week's collection and washings, preserved with a little formaldehyde, were dried on the steam bath at the end of the week, after which the dried material was ground, put through a 20 mesh sieve, and weighed. Samples from this collection were then weighed out for analysis.

Sodium in the feed, feces, and urine was determined essentially by the method of Tisdall and Kramer (9). The only change adopted was that porcelain dishes were used for most of the deter-

minations in place of platinum ones. This method was tested out by running blank determinations in the porcelain dishes. The net changes in weight of crucibles which received washings from the

TABLE II.

Sodium and Chlorine Balance Data on Rats on the Daily Basis.

Rat No.	Ration.	Sodium. Intake.	Sodium. Output urine.	Sodium. Output feces.	Sodium. Total output.	Sodium. Balance.	Chlorine. Intake.	Chlorine. Output urine.	Chlorine. Output feces.	Chlorine. Total output.	Chlorine. Balance.
Rats 17 weeks old. Food intakes of pair mates the same.											
		mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
1	A	4.9	17.0	0.5	17.5	−12.6	4.6	5.8	0.2	6.0	−1.4
2	B	62.2	59.7	2.6	62.3	−0.1	90.5	84.9	1.3	86.2	+4.3
3	A	4.3	12.9	0.3	13.2	−8.9	4.0	7.3	0.3	7.6	−3.6
4	B	62.2	56.2	1.4	57.6	+4.6	90.5	89.3	1.7	91.0	−0.5
5	A	4.1	17.0	0.3	17.3	−13.2	3.8	13.5	0.2	13.7	−9.9
6	B	51.8	50.6	1.3	51.9	−0.1	75.4	75.4	1.3	76.7	−1.3
7	A	4.1	12.7	0.6	13.3	−9.2	3.8	16.9	0	16.9	−13.1
8	B	49.5	47.3	2.1	49.4	+0.1	72.1	64.7	1.0	65.7	+6.4
Rats 9 weeks old. Food intakes of pair mates approximately the same per unit of body surface.											
9	A	4.3	21.9	0.3	22.2	−17.9	4.2	4.9	0.2	5.1	−0.9
10	B	49.9	47.9	1.7	49.6	+0.3	66.4	51.9	0.7	52.6	+13.8
11	A	4.3	19.5	0.4	19.9	−15.6	4.2	5.3	0.1	5.4	−1.2
12	B	49.9	45.9	1.2	47.1	+2.8	66.4	48.3	1.1	49.4	+17.0
13	A	4.1	19.9	0.3	20.2	−16.1	4.0	5.3	0.3	5.6	−1.6
14	B	49.9	46.8	2.2	49.0	+0.9	66.4	51.8	1.2	53.0	+13.4
15	A	3.7	17.6	0.4	18.0	−14.3	3.6	6.2	0	6.2	−2.6
16	B	49.9	52.1	1.3	53.4	−3.5	66.4	50.8	1.1	51.9	+14.5

dishes were + 0.0009 gm., − 0.0001 gm., − 0.0008 gm., and + 0.0001 gm. It was therefore concluded that the use of porcelain dishes does not lead to the contamination of the analytical samples with sodium.

Chlorine was determined according to the Volhard volumetric method, described in the Methods of Analysis of the Association of Official Agricultural Chemists, with slight modifications. The

TABLE III.

Nitrogen Balance Data on Rats.

Rat No.	Ration.	Nitrogen.					
		Intake.	Output urine.	Output feces.	Total output.	Balance.	Coefficient of digestibility.
Rats 17 weeks old. Food intakes of pair mates the same.							
		mg.	*mg.*	*mg.*	*mg.*	*mg.*	
1	A	300	210	28	238	+62	90.6
2	B	307	214	36	250	+57	88.1
3	A	263	202	29	231	+32	88.8
4	B	307	206	44	250	+57	85.6
5	A	250	205	25	230	+20	89.9
6	B	256	205	28	233	+23	88.9
7	A	250	222	23	245	+5	91.8
8	B	245	191	26	217	+28	89.4
Rats 9 weeks old. Food intakes of pair mates approximately the same per unit of body surface.							
9	A	236	166	25	191	+45	89.5
10	B	257	157	26	183	+74	90.0
11	A	235	169	21	190	+45	91.2
12	B	257	146	26	172	+85	89.8
13	A	226	152	25	177	+49	89.0
14	B	257	150	23	173	+84	91.1
15	A	204	151	20	171	+33	90.3
16	B	257	177	30	207	+50	88.1

silver nitrate used was approximately N/20 and the ammonium sulfocyanate used approximately N/40, modifications suggested by Halverson and Wells (10).

The chlorine, sodium, and nitrogen balance data for the rats

TABLE IV.

Sodium and Chlorine Balance Data on Chicks.

Chick No.	Ration.	Sodium.			Chlorine.		
		Intake.	Output.	Balance.	Intake.	Output.	Balance.
		mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
1	A	12.3	6.9	+5.4	9.1	4.1	+5.0
2	B	105.8	77.6	+28.2	187.1	90.5	+86.9
3	A	9.6	3.7	+5.9	7.1	2.0	+5.1
4	B	87.8	65.0	+22.8	155.6	82.7	+72.9
5	A	12.1	7.4	+4.7	8.9	6.2	+2.7
6	B	105.6	80.0	+25.6	187.1	99.7	+87.4
7	A	12.2	4.2	+8.0	9.0	3.7	+5.3
8	B	104.2	76.0	+28.2	184.6	103.4	+81.2

TABLE V.

Nitrogen Balance Data on Chicks and Metabolizable Energy Determinations of the Feed.

Chick No.	Ration.	Nitrogen.			Energy.				
		Intake.	Output.	Balance.	Intake.	Output.	Correction for gain of protein	Metabolizable energy consumed daily.	Metabolizable energy per gm. of food.
		mg.	*mg.*	*mg.*	*cals.*	*cals.*	*cals.*	*cals.*	*cals.*
1	A	635	420	+215	102.2	18.2	1.8	82.2	3.39
2	B	645	322	+323	104.3	14.8	2.3	87.2	3.50
3	A	495	356	+139	79.7	14.1	1.0	64.6	3.42
4	B	535	300	+235	86.6	13.9	1.7	71.0	3.44
5	A	626	358	+268	100.8	17.1	1.9	81.8	3.43
6	B	644	348	+296	104.1	15.7	2.1	86.3	3.47
7	A	630	433	+197	101.6	19.5	1.4	80.7	3.35
8	B	635	346	+289	102.7	19.7	2.1	80.9	3.30

are summarized in Tables II and III, while similar data for the chicks will be found in Tables IV and V. For the chicks, the gross energy content of feed and excreta was directly determined in the bomb calorimeter.

DISCUSSION OF RESULTS.

Growth Observations.—An inspection of the growth curves shows that without exception, the rats receiving the added NaCl made more rapid gains in weight than their mates receiving no added NaCl, though toward the end of the experiment there was a tendency for the rats getting Ration A to overtake those getting Ration B. The explanation for this may be that the animals receiving sodium chloride grew faster and their maintenance requirements were thus increased. Consequently, when restricted to the same amounts of food as their lighter mates, they were less adequately nourished with respect to energy. In the later weeks of the experiment this handicap more than compensated for the superior growth-promoting value of Ration B over Ration A. Rats 9 to 16 inclusive, whose food was controlled in proportion to the two-thirds power of the body weight, naturally do not exhibit this tendency so much, and the difference between the growth of pair mates was greater and more prolonged. However, it should be pointed out that toward the end of the experiment, the rats on Ration B refused to eat as much in proportion to the two-thirds power of their weight as the rats getting Ration A.

The total gains in weight of Rats 2, 4, and 6, receiving added NaCl in their food, averaged 43 per cent greater than those of their pair mates, getting the same amount of food. Rats 7 and 8 showed about the same total gains, being the only pair of animals showing no clear effect of the NaCl supplement. Rats 10, 12, 14, and 16, receiving NaCl, made total gains averaging 89 per cent greater than the gains made by their pair mates receiving no NaCl but slightly more total food in proportion to the two-thirds power of the weight.

With all pairs of rats, the amount of food required to produce a gm. gain in weight was less for the NaCl rats than for the controls, quite distinctly less with the one exception above noted. Omitting Rats 7 and 8, the average amount of food consumed per gm. of gain produced was 5.6 gm. for the NaCl rats and 7.6 gm. for the control rats.

The growth data on the chicks show as striking differences between animals getting NaCl and those not getting NaCl as do the data on the rats. For the 12 weeks of the experiment, the NaCl chicks gained 55 per cent faster than the control chicks, each pair of chicks showing a distinct difference in this respect. The average amount of food required per gm. of gain was 4.5 gm. for the NaCl chicks and 6.9 gm. for the control chicks getting no added NaCl.

The growth data of this experiment afford a striking demonstration of the fact that the utilization of food energy by growing animals may be greatly impaired by an improper balance among indispensable dietary factors. The addition of 1 per cent of NaCl to Ration A has evidently greatly improved the utilization of its available energy, probably by decreasing its stimulating effect on heat production, *i.e.* its specific dynamic effect, and possibly to some extent by decreasing the muscular activity or restlessness of the rat itself.

An inspection of the growth curves of the rats fed by Osborne and Mendel (6) on rations low in (*a*) sodium, (*b*) sodium, chlorine, and magnesium, and (*c*) chlorine, shows gains of about 185 and 115 gm. (low sodium), 130 and 110 gm. (low sodium, chlorine, and magnesium), and 168 and 78 gm. (low chlorine) in the first 80 days of the experiment, a period approximately the length of the growth experiment reported in this paper. These rats were all males from 70 to 90 gm. in weight at the beginning of the experiment and can thus be compared with the five males in the present investigation that were on Ration A.

The five males receiving Ration A in our experiment made the following gains in 82 to 84 days from initial weights of 54 to 68 gm.: 181, 118, 108, 106, and 83 gm.

These rats made approximately as good gains as four of the six rats in Osborne and Mendel's experiments, receiving diets low in sodium or chlorine or both. Their pair mates, however, receiving Ration B, made much better growth, showing that the rats on Ration A were not growing at their maximum rate. This raises the question whether four of the six rats in Osborne and Mendel's experiments on rations comparable to ours also were not growing as well as they might have grown if salt had been included in the ration, and hence were not growing at their maximum rate. If

this is the case, it cannot be concluded that their growth was not limited by a deficient content of mineral elements in the ration. The two rats which made such rapid gains on the low sodium and low sodium and low chlorine diets, respectively, appear to be exceptions, possibly due to an unusually large consumption of food. Even their growth, however, was not as rapid as that of most of the male rats receiving added NaCl in our experiment, though the food intake of these rats was restricted.

The male rats receiving Ration B made gains much superior to the so called "normal" curves of either Osborne and Mendel or Donaldson, attaining weights of from 225 to 265 gm. at the age of about 120 days. The normal curve of Donaldson shows that at this age the normal male weighs about 190 gm.

Balance Data.—On inspecting the chemical data obtained in the balance periods with the rats and chickens, the following facts are apparent.

1. Both rats and chickens on Ration B gave distinctly more favorable balances of sodium and chlorine than their pair mates on Ration A low in these elements. This is true of all pairs of animals.

2. The rats on Ration A all showed considerable negative sodium balances, while the rats on Ration B were in approximate sodium equilibrium.

3. The rats on Ration A were, with two exceptions, in approximate chlorine equilibrium, while those on Ration B showed an appreciable positive balance with two exceptions.

4. The chicks on both Rations A and B were in positive balance as regards sodium, but those on Ration B showed much larger positive balances.

5. The chicks on both rations showed positive chlorine balances, but those on Ration B were retaining daily much larger amounts of chlorine.

6. While the daily retention of chlorine by the chicks on Ration A was about the same as the daily retention of sodium, on Ration B the daily retention of chlorine was approximately three times that of sodium.

7. The nitrogen balances are generally in accord with the growth curves; that is, with one exception, the animals on Ration B made

more rapid growth than their pair mates and also showed a greater retention of nitrogen.

Regarding the first statement made above, there is little to be said. The fact that in every case the animal receiving Ration B showed a more favorable balance of both sodium and chlorine indicates that corn is deficient in both of these elements, unless their utilization in the body is largely as NaCl. However, the fact that the daily losses of chlorine on Ration A were generally smaller with the rats than the daily losses of sodium, while on Ration B the chlorine balances were generally distinctly more favorable than the sodium balances, suggests that the sodium deficiency of corn is more pronounced than its chlorine deficiency. This conclusion is supported by the data on the chickens. Although on Ration A, the daily balances of sodium and of chlorine were about the same, on Ration B the chicks stored approximately three times as much chlorine as sodium, quite out of proportion to their relative atomic weights.

That the negative sodium balances of the rats on Ration A could be typical of the entire period of 82 to 84 days is scarcely conceivable, since all the rats grew, even though slowly, throughout the experiment. Obviously the short balance periods were not typical of the entire growth experiment. The results of these balance periods are, therefore, of comparative rather than absolute value.

The metabolism results on the chickens are evidently more representative of the period of growth than the results with the rats. All of the birds were retaining some sodium and chlorine—those on Ration B quite large amounts. Since all of the birds were gaining in weight, this is what one might expect if sodium and chlorine were being used in the production of new tissue. On the other hand, if these two elements were merely being used temporarily in the body fluids, it might be expected that they would be excreted as rapidly as they were consumed, thus putting the animal in approximate equilibrium regardless of the intake of the elements.

All animals on Ration B with one exception were retaining nitrogen at a more rapid rate than their pair mates on Ration A. Evidently the utilization of the nitrogen of Ration A as well as of its energy was enhanced by the addition of sodium chloride.

One point that should be brought out in connection with the nitrogen balance figures on the rats is that the rats on both rations digested the nitrogen of their food about equally well, the average coefficient for Ration A being 90.1, and for Ration B, 88.9. If the cause for the poorer growth of the animals on Ration A were due to failure to secrete sufficient hydrochloric acid in the gastric juice, one might expect a much lower coefficient of digestibility of the protein of the ration. However, such was not the case.

The data contained in Table V relative to the energy content of food and excreta of the chicks during their metabolism period also indicate no significant impairment of digestive capacity on the ration deficient in sodium and chlorine. The chicks on Ration A obtained 3.40 calories of metabolizable energy from each gm. of food consumed, while the chicks on Ration B obtained 3.43 calories of metabolizable energy per gm. of food consumed. Ration A contained 4.22 calories of gross energy per gm., so that its gross energy proved to be 80.6 per cent metabolizable. Ration B contained 4.19 calories of gross energy per gm., of which 81.9 per cent was metabolizable.

CONCLUSIONS.

From the above experimental findings it may be concluded:

1. The addition of sodium chloride to a ration composed largely of corn, containing 0.047 per cent sodium and 0.041 per cent chlorine, enhances its growth-promoting value.

2. Probably the concentration of both sodium and chlorine in corn is too low to promote maximal growth in rats and chickens. However, there are indications in the balance data that the sodium deficiency of corn is greater than its chlorine deficiency, in the sense that considerably more of sodium than of chlorine is required to supplement it completely.

3. The deficiency of corn in sodium and chlorine limits the utilization of its energy and protein for growth.

4. On a ration composed largely of corn, unsupplemented by sodium chloride, the digestibility of protein is not impaired by a deficiency of hydrochloric acid in the gastric juice. The deficiency of chlorine seems to be related more to the requirements for growth than to the requirements for gastric secretion. Furthermore, the

metabolizable energy derivable from a given weight of corn is not lowered by the sodium and chlorine deficiency of such a ration.

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THE BIOLOGICAL VALUE OF THE NITROGEN OF MIXTURES OF PATENT WHITE FLOUR AND ANIMAL FOODS.

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The peculiar function of protein and protein derivatives in the body, which, in practical nutrition, cannot be served by any other nutrient, is to provide structural units (amino acids) for the synthesis of the nitrogenous constituents, protein and non-protein in character, of the tissues and secretions of the body. These constituents are extremely varied in character, but it is fair to assume, since the assumption does not contradict any of the known facts or the accepted theories of physiology, that the proportions of these constituents that are catabolized endogenously and that must be replaced in order to maintain the integrity of the tissues, are fairly constant for different conditions of the environment or of the animal itself. The known constancy of the creatinine excretion in the urine speaks for the correctness of this assumption. Furthermore, it is known that the proportions of the different nitrogenous constituents needed in the formation of new tissue in growth and reproduction, and in the elaboration of milk during lactation, are very little affected by conditions of feeding, activity, or variable environmental factors.

Hence, though the percentage of nitrogen in the different nitrogenous compounds of the body varies greatly, the need for them, or for their precursors (dietary amino acids), may fairly be measured by the total nitrogen content of the tissue constituents catabolized endogenously (maintainance), or by the total nitrogen content of the new tissues formed in growth and reproduction, or by the total nitrogen content of the milk produced in lactation.

A complete determination of the "protein value" of a food or of a mixture of foods in covering these nitrogen requirements of ani-

mals must consider (1) the total nitrogen content of the food or ration, (2) the loss or wastage of nitrogen in digestion, and (3) the loss or wastage of nitrogen in the process of its conversion into tissue constituents or body secretions.

It is possible to determine the true protein content of foods and rations by laborious chemical methods, but in the routine analysis of foods the determination of protein is based solely upon the nitrogen content, which when multiplied by a conventional factor (6.25) is called the "crude protein" content. This procedure was originally introduced as a purely chemical expedient, but after all it would appear to possess more significance from a biological standpoint than the true protein content itself. The non-protein nitrogenous constituents of foods are, in the main, amino acids or simple derivatives of amino acids, which may, in all probability, participate to a significant extent in the synthesis of the nitrogenous constituents of animal tissues and secretions, either individually, or in conjunction with the end-products of protein digestion. They should not, therefore, be excluded from consideration in the determination of the protein value of foods and rations. For this reason, the total nitrogen content of a food or mixture of foods is a better starting point in this determination than the total content of true protein.

Another argument for this conception is the fact that the losses of protein, or, more appropriately, amino acids, in digestion and metabolism, can only be measured in terms of nitrogen. Coefficients of digestibility of proteins and biological values of proteins are necessarily based solely upon nitrogen determinations, and refer solely to the utilization of food nitrogen in the animal body. They are therefore of significance only with reference to the total nitrogen content of the food or ration with which they have been obtained. While, therefore, in the chemical characterization of a food or ration, the true protein content, referring to a definite class of chemical compounds, possesses a much greater significance than the content of crude protein, in the biological evaluation of the food or ration, the total nitrogen content, converted into a purely conventional protein term, *i.e.*, crude protein, must be given the preference.

Therefore, both in the determination of the protein values of foods and in the determination of the so called "protein" requirements of animals, it seems advisable to speak in terms of nitrogen.

According to numerous dietary studies made by the U. S. Department of Agriculture and summarized by Langworthy in Circular 110 of the Office of Experiment Stations, white flour contributes almost 30 per cent of the protein (nitrogen) in the average American dietary. Other cereal preparations contribute about 13 per cent, while the legumes contribute about 3 per cent of the dietary protein. The nitrogen of these foods possesses relatively low biological values, much lower than the nitrogen in staple animal foods.

The addition of animal foods to such staple plant foods as those just mentioned, would conceivably correct the protein value of the diet in two distinct ways; first, by providing additional nitrogen of high biological value, and second, by supplementing the plant nitrogen and increasing in this way its value to the body. In so far as this supplementing action is based upon the amino acid make-up of the different classes of foods, it would be expected to appear whenever the amino acids constituting the limiting factors in the utilization of the two sources of nitrogen to be mixed, are not the same. If the indispensable amino acid limiting the utilization of the nitrogen of meat, for example, is not the limiting factor for the nitrogen of white flour, it is evident that if the two foods are fed together, the nitrogenous compounds of meat will enhance the biological value of the nitrogenous compounds of white flour, and also that the nitrogenous compounds of white flour will enhance the biological value of the nitrogenous compounds of meat. Only when the limiting amino acid is the same for both flour and meat will no supplementing relation exist. On the other hand, the supplementing effect may not be great, even if present, since, when the first limiting deficiencies of the two sources of nitrogen are mutually satisfied, the utilization of the mixture may still be seriously limited by the relative deficiency in each individual food of some common indispensable amino acid.

In determining the value in the human diet of food products of animal origin, we have been interested not only in the inherent biological value of the nitrogen of such foods, but also in the extent to which their nitrogenous constituents are capable of supplementing the nitrogenous constituents of staple cereal foods, themselves possessing low biological values. Among these foods, patent white flour is the most important quantitatively and, therefore, has received our first attention. The plan of the experi-

ments to be reported in this paper has been to determine in the same experiment and with the same experimental animals, the biological value of the nitrogen of the animal food alone, of white flour alone, and of a mixture of the two, always in the proportion of one part of nitrogen from the animal food to two parts of nitrogen from white flour. Each test has generally been conducted upon ten rats handled in two groups of five. The nitrogen balance determinations on these two groups of animals were made by different persons and the order in which the foods to be tested were fed was different for the two groups.

The details of the experiments relative to the preparation of rations, the method of feeding, and the methods of collecting the

TABLE I.

Composition of Rations.

Ingredients.	White flour ration.	Egg ration.	Low N ration.
Wheat flour	70		
Dried egg		17	
Butter fat	8	8	8
Cod liver oil	2	2	2
Salt mixture	3	3	4
Starch	5	58	74
Sucrose	10	10	10
Agar	2	2	2

excreta were similar to those already described in earlier reports of similar experiments (1) from this laboratory.

I. White Flour and Whole Egg.

The first combination of foods tested in this study was white flour and whole egg. The composition of the rations used in this test is given in Table I. The white flour was a standard brand in the midwest. The dried egg was prepared in the laboratory from eggs obtained from the Poultry Division of the Agricultural Experiment Station. The butter fat was prepared from University butter by melting and centrifuging to remove the casein. The cod liver oil was the product put out for animal feeding by the E. L. Patch Co. The salt mixture was prepared according to the directions of Osborne and Mendel (2). In this, as in all later

experiments, each rat received daily, besides its allotment of these rations, 25 mg. of yeast vitamin (Harris) as a source of vitamin B. This amount of vitamin concentrate contained from 2 to 3 mg. of nitrogen, which has been entirely neglected in the calculations based on the nitrogen metabolism data. The mixed ration used in this experiment was prepared by combining the white flour and the egg rations in the ratio of 2 to 1.

The complete data of the nitrogen metabolism periods with the intermediate calculations leading to the final biological values for the first five rats are contained in Table II. The experimental data for the other five rats are given in Table III. In this and all subsequent tables, the intermediate calculations leading to the final biological value will be omitted.

The biological values calculated for these different rations presumably represent the percentage of the absorbed nitrogen used by growing rats for both maintenance and growth. Their calculation involves the assumption that the excretion of fecal nitrogen per gm. of food consumed on the low nitrogen ration in the first and final periods measures the excretion of body nitrogen in the feces in the intervening periods. The change in the excretion of the fecal nitrogen per gm. of low nitrogen ration from the first to the sixth period is assumed to occur in a linear fashion with respect to time. The second assumption involved in these calculations is that the excretion of nitrogen in the urine per 100 gm. of body weight in the first and final periods of low nitrogen feeding is a measure of the excretion of body nitrogen in the urine in the intervening periods, the change in these values from the first to the sixth period being also assumed to be linear.

If the estimated daily quantity of body nitrogen in the feces exceeds the total fecal nitrogen actually found, as frequently occurred in many of these experiments, it has been assumed that all of the food nitrogen is digestible. Also, with Rats 226 to 230, inclusive, on the whole egg ration, the estimated quantity of body nitrogen in the urine generally exceeded the actual total urinary nitrogen. This is interpreted to mean that no food nitrogen in these periods was wasted in the urine, so that the absorbed nitrogen was utilized to the extent of 100 per cent. This is a very rare occurrence, however, which has not been shown in any experiment but the one mentioned above.

TABLE II.

Nitrogen Metabolism Data and the Calculation of Biological Values for the First Group of Rats.

Rat No.	Body weights. Initial.	Body weights. Final.	Food intake.	N intake.	Fecal N.	Body N in feces.	Food N in feces.	Absorbed N.	N in urine.	Body N in urine.	Food N in urine.	Food N retained.	Biological value.
	gm.	*gm.*	*gm.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*per cent*
Period 1. Low nitrogen ration (0.016 per cent N).													
221	173	163	10.00		17	1.7*			32	19†			
222	190	175	8.10		14	1.8*			42	23†			
223	171	158	9.03		15	1.6*			42	25†			
224	185	173	8.13		14	1.7*			39	22†			
225	172	163	9.81		18	1.9*			38	23†			
Period 2. Egg ration (1.27 per cent N).													
221	188	207	10.00	127	22	18	4	123	58	34	24	99	81
222	196	217	10.00	127	20	18	2	126	48	43	5	121	96
223	179	199	10.00	127	20	17	3	125	54	43	11	114	91
224	199	219	10.00	127	22	17	5	123	52	41	11	112	91
225	183	209	10.00	127	16	19	0	127	52	41	11	116	91
Period 3. Flour, egg ration (1.28 per cent N).													
221	215	223	9.95	127	18	19	0	127	82	33	49	78	62
222	222	229	9.49	121	13	18	0	121	81	43	38	83	69
223	205	214	10.00	128	17	18	0	128	79	42	37	91	71
224	221	235	9.86	126	14	17	0	126	74	40	34	92	72
225	215	222	10.00	128	17	20	0	128	79	41	38	90	70
Period 4. Flour ration (1.20 per cent N).													
221	225	227	10.00	120	18	20	0	120	96	31	65	55	46
222	229	232	10.00	120	17	20	0	120	97	39	58	62	52
223	210	215	10.00	120	16	19	0	120	97	37	60	60	49
224	233	235	10.00	120	15	18	0	120	101	36	65	55	46
225	219	222	10.00	120	16	21	0	120	99	37	62	58	48
Period 5. Flour ration (1.29 per cent N).													
221	232	234	10.00	129	18	21	0	129	100	26	74	55	43
222	237	238	10.00	129	14	21	0	129	94	35	59	70	54
223	219	226	10.00	129	14	20	0	129	99	31	68	61	47
224	240	244	10.00	129	14	18	0	129	97	31	66	63	49
225	226	228	10.00	129	17	22	0	129	101	33	67	62	47

TABLE II—*Concluded.*

Rat No.	Body weights. Initial.	Body weights. Final.	Food intake.	N intake.	Fecal N.	Body N in feces.	Food N in feces.	Absorbed N.	N in urine.	Body N in urine.	Food N in urine.	Food N retained.	Biological value.
Period 6. Low nitrogen ration (0.025 per cent N).													
	gm.	*gm.*	*gm.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*per cent*
221	232	220	8.35		18	2.2*			22	9†			
222	238	221	6.70		15	2.2*			30	13†			
223	221	204	7.94		17	2.1*			24	11†			
224	243	232	8.84		16	1.8*			25	11†			
225	225	212	8.18		19	2.3*			27	12†			

* These figures represent the metabolic fecal nitrogen per gm. of food consumed and are used in estimating the body nitrogen in the feces in the intermediate periods from the amount of food consumed.

† These figures represent the endogenous urinary nitrogen per 100 gm. of body weight and are used in estimating the body nitrogen in the urine in the intermediate periods from the average body weight.

With each of the two groups of rats in this experiment, either the white flour ration or the whole egg ration was fed in two consecutive periods to see how close a check could be obtained in the estimated biological value of the food nitrogen. With Rats 221 to 225, inclusive, very close checks were obtained between these two periods of like feeding, the average biological value for the two periods being identical. With Rats 226 to 230, inclusive, a good check was also obtained with reference to the whole egg ration, in spite of the fact that with four of the five rats, the amount of food consumed was approximately 1 gm. more per day in the second period than in the first. The duplicate biological values for each of the rats are as follows:

White flour nitrogen.			Whole egg nitrogen.		
Rat No.	Period 4.	Period 5.	Rat No.	Period 4.	Period 5.
221	46	43	226	100	100
222	52	54	227	100	100
223	49	47	228	100	100
224	46	49	229	99	95
225	48	47	230	100	100
Average...	48	48		100	99

A summary of the biological values obtained for the ten rats in this test is given in Table IV. It is evident that the second group of rats utilized their dietary nitrogen consistently better than the first group of rats. The biological value for egg nitrogen previously reported (3) was 93, very close to the average for the ten rats in this test.

In the last column of Table IV, the biological value of the mixed ration has been estimated from the biological values found for the

TABLE III.

Nitrogen Metabolism Data for the Second Group of Rats.

Rat No.	Body weights. Initial.	Body weights. Final.	Food intake.	N intake.	Fecal N.	N in urine.	Body weights. Initial.	Body weights. Final.	Food intake.	N intake.	Fecal N.	N in urine.
	Period 1. Low nitrogen ration (0.016 per cent N).						**Period 4. Egg ration (1.27 per cent N).**					
	gm.	*gm.*	*gm.*	*mg.*	*mg.*	*mg.*	*gm.*	*gm.*	*gm.*	*mg.*	*mg.*	*mg.*
226	113	98	4.66		9	26	132	151	7.99	102	14	26
227	144	133	10.00		17	29	176	196	8.99	114	14	32
228	110	96	5.69		11	28	142	160	8.00	102	13	27
229	100	89	5.37		11	23	130	149	7.99	102	12	29
230	105	94	5.23		13	25	138	159	8.00	102	12	29
	Period 2. Flour ration (1.29 per cent N).						**Period 5. Egg ration (1.27 per cent N).**					
226	117	114	6.05	78	14	59	158	172	8.02	102	18	32
227	146	156	8.91	115	13	85	202	226	9.97	127	18	38
228	116	124	7.88	102	11	78	166	185	8.99	114	14	36
229	102	113	7.34	95	12	65	156	174	8.98	114	15	36
230	107	114	7.32	95	11	68	164	185	8.99	114	18	36
	Period 3. Flour, egg ration (1.28 per cent N).						**Period 6. Low nitrogen ration (0.025 per cent N).**					
226	119	122	6.15	79	11	42	170	150	5.21		12	31
227	161	168	8.94	115	16	42	217	191	5.01		13	38
228	127	137	7.93	102	12	57	178	154	4.33		10	37
229	115	123	7.35	94	11	53	172	153	5.60		16	26
230	120	130	7.81	100	12	50	179	157	4.16		11	34

two rations separately and from the proportions in which they were combined. These estimated values may be taken as the values that would have been obtained for the mixed ration if no supplementary relation existed between the nitrogen compounds in white flour and the nitrogen compounds in whole egg. However, for each of the ten rats the estimated biological value of the mixed ration is less than the value actually obtained, indicating that an appreciable supplementary relation exists between these two sources of nitrogen. As an average for the first group of rats the actual biological value obtained for the mixture was 7 per cent

TABLE IV.

Summary of Biological Values Obtained.

Rat No.	White flour ration.	Egg ration.	Mixture.	Estimated values if no supplementing effect existed.
221	44	81	62	56
222	53	96	69	67
223	48	91	71	62
224	47	91	72	62
225	47	91	70	62
Average	48	90	69	62
226	58	100	83	72
227	53	100	92	69
228	54	100	77	69
229	57	97	71	70
230	57	100	80	71
Average	56	99	81	70

greater than the value to be expected if no supplementary action occurred. With the second group of rats the average increase was 11 per cent.

II. Flour and Egg Albumin.

The next experiments were designed to obtain information concerning the biological value of the nitrogen of egg albumin and egg yolk, separately, and each combined with white flour nitrogen in the proportion of 1 to 2. Although very constant values were obtained for egg albumin among the different groups of rats experi-

mented upon, an unaccountably wide variability existed among the values obtained for the egg yolk fed under analogous conditions. For a number of groups of rats giving consistent values for egg albumin, the values obtained for egg yolk varied all the way from 75 to 100 per cent, although the ration was consumed readily and in adequate amounts and no evidence was apparent during the course of the experiments for the presence of any abnormal conditions. It was hardly thought worth while, therefore, to present such a series of discordant values for this fraction of the hen's egg. We hope to continue experimental work on egg yolk nitrogen.

TABLE V.

Composition of Rations.

Ingredients.	Egg albumin ration.	White flour ration.	Low egg-nitrogen ration.
Dried egg albumin	10.3	0	0
" whole egg	0	0	8.5
White flour	0	70	0
Cellu " *	4	4	4
Sucrose	10	10	10
Corn starch	61.7	2	63.5
Butter fat	10	10	10
Salt mixture	4	4	4

* A product obtained from the Chicago Dietetic Supply House, containing, by actual analysis, 37.8 per cent crude fiber and only 0.015 per cent of nitrogen.

The composition of the rations used in these experiments is given in Table V. It will be noted that in place of a ration containing no nitrogenous nutrients, we have used in these experiments a ration containing a small amount of dried whole egg, the concentration being equivalent to about 4 per cent of crude protein. There has existed in this type of work some objection to the use of a ration containing no nitrogenous materials in the initial and final periods. In such periods of nitrogen-free feeding, the animals invariably lose in weight, and, in the last days of experimental feeding, frequently leave considerable food residues, so that the average consumption of food per day for these periods has generally been distinctly less than for the intervening test periods. A

subnormal food consumption in these periods, in which the rat is standardized relative to the nitrogen of body origin in urine and feces, throws some doubt on the applicability of the results obtained to other periods in which the food consumption is distinctly larger. The nitrogen of dried whole egg is apparently completely digestible, and, in the low concentration used in this low egg-nitrogen ration, is probably completely utilized in metabolism. When fed at twice the concentration used in this ration, its biological value has been found to average approximately 94 per cent. For a lower concentration, this value would presumably increase (4). Direct testimony on these points will be presented in the following section of this paper.

Another slight departure in the make-up of these rations as compared with the rations heretofore used, is the inclusion in all rations of equal amounts of a source of roughage. We have used for this purpose a commercial product known as Cellu Flour, which is practically nitrogen-free and which contains about 38 per cent of crude fiber.

The results of the nitrogen metabolism periods are given in Table VI, and the biological values computed from them are summarized in Table VII. The biological values obtained for egg albumin nitrogen are very concordant with the exception of that for one rat, No. 263, for which an abnormally low value was obtained; the average values obtained for the four groups of rats fed the egg albumin ration are 86, 81, 81, and 82.

The biological values for white flour nitrogen for the two groups of rats fed upon the white flour ration are not as concordant as the values obtained with egg albumin, the averages being 48 and 59. For the mixed ration, the individual values agreed satisfactorily, averaging 65 for the first group of rats, and 67 for the second group of rats.

The computed biological values for the mixed ration, given in the last column of Table VII, were, in general, slightly lower than the values found experimentally, but in several cases the computed and found values were either the same, or the computed values were larger than the found values, this being especially true in the second group of rats, Nos. 260 to 264 inclusive. Possibly with this group of rats the high values obtained with the white flour ration were abnormally high, so that actually a greater supplementary

TABLE VI.

Nitrogen Metabolism Data.

Rat No.	Body weights. Initial.	Body weights. Final.	Food intake.	N intake.	Fecal N.	N in urine.	Body weights. Initial.	Body weights. Final.	Food intake.	N intake.	Fecal N.	N in urine
	Period 1. Low egg-nitrogen ration (0.732 per cent N).						Period 4. Flour ration (1.53 per cent N).					
	gm.	*gm.*	*gm.*	*mg.*	*mg.*	*mg.*	*gm.*	*gm.*	*gm.*	*mg.*	*mg.*	*mg.*
250	100	109	9.00		11	21	148	145	7.07	108	16	79
251	111	119	9.00		10	23	160	165	9.00	137	15	104
252	103	112	9.00		10	22	152	157	9.00	137	14	100
253	101	110	9.00		11	21	152	155	8.94	136	20	97
254	102	111	9.00		10	20	148	153	8.59	131	15	92
	Period 2. Egg albumin ration (1.43 per cent N).						Period 7. Low egg-nitrogen ration (0.732 per cent N).					
250	120	131	8.81	126	11	40	184	185	8.19		14	25
251	130	143	9.00	129	9	46	203	203	9.00		17	34
252	122	136	9.00	129	9	46	200	200	9.00		14	38
253	120	135	8.93	128	13	41	200	199	9.00		*	32
254	120	131	8.27	118	9	40	194	192	9.00		*	40
	Period 3. Egg albumin, flour ration (1.48 per cent N).											
250	133	141	8.28	123	13	68						
251	145	155	9.00	133	10	76						
252	137	145	9.00	133	12	73						
253	135	146	9.00	133	14	70						
254	132	141	8.57	127	13	69						
	Period 1. Low egg-nitrogen ration (0.732 per cent N).						Period 6. Egg albumin ration (1.43 per cent N).					
260	97	105	9.00		9	24	169	181	8.85	127	10	53
261	89	98	9.00		11	27	168	175	8.08	116	12	64
262	92	102	9.00		9	22	164	176	9.00	129	11	62
263	94	100	9.00		12	22	162	169	8.38	120	12	74
264	87	95	9.00		11	26	155	162	7.58	108	10	62

* Because the fecal nitrogen determinations in these cases were lost, the average amount of fecal nitrogen per gm. of food consumed for the other three rats in the group was assumed to apply to these rats.

TABLE VI—*Continued.*

Rat No.	Body weights. Initial.	Body weights. Final.	Food intake.	N intake.	Fecal N.	N in urine.	Body weights. Initial.	Body weights. Final.	Food intake.	N intake.	Fecal N.	N in urine.
	Period 4. Flour ration (1.53 per cent N).						Period 7. Low egg-nitrogen ration (0.732 per cent N).					
	gm.	*gm.*	*gm.*	*mg.*	*mg.*	*mg.*	*gm.*	*gm.*	*gm.*	*mg.*	*mg.*	*mg.*
260	154	156	8.04	123	17	87	184	185	9.00		9	37
261	155	158	8.00	122	†	87	175	180	9.00		14	43
262	148	153	9.00	137	19	94	175	175	9.00		14	44
263	151	152	8.14	124	†	83	170	171	9.00		15	39
264	148	149	8.58	131	13	80	163	162	8.87		15	43
	Period 5. Flour, egg albumin ration (1.48 per cent N.)											
260	158	166	8.97	133	16	81						
261	158	165	8.21	122	13	77						
262	154	162	9.00	133	15	83						
263	151	158	8.13	121	11	76						
264	148	158	8.98	133	13	84						
	Period 1. Low egg-nitrogen ration (0.679 per cent N).						Period 5. Low egg-nitrogen ration (0.679 per cent N).					
270	88	96	8.47		13	17	151	153	8.50		14	32
271	72	80	7.82		12	15	140	138	7.41		14	26
272	74	77	6.75		12	15	136	132	6.13		12	24
273	76	80	7.01		13	15	123	123	6.65		12	26
274	75	82	8.23		13	15	144	142	7.39		14	28
	Period 3. Egg albumin ration (1.37 per cent N).											
270	126	136	8.50	117	13	45						
271	109	120	8.50	117	11	42						
272	108	118	8.50	117	13	50						
273	107	114	8.06	111	12	46						
274	114	124	8.50	117	11	41						

† Since the fecal nitrogen determinations were lost for these rats, an average digestibility of the dietary nitrogen was assumed.

TABLE VI—*Concluded.*

Rat No.	Body weights. Initial.	Body weights. Final.	Food intake.	N intake.	Fecal N.	N in urine.	Body weights. Initial.	Body weights. Final.	Food intake.	N intake.	Fecal N.	N in urine
	Period 1. Low egg-nitrogen ration (0.679 per cent N).						Period 5. Low egg-nitrogen ration (0.679 per cent N).					
	gm.	*gm.*	*gm.*	*mg.*	*mg.*	*mg.*	*gm.*	*gm.*	*gm.*	*mg.*	*mg.*	*mg.*
280	73	78	7.01		12	14	143	142	7.05		13	28
281	84	94	8.03		13	15	160	163	8.91		10	29
282	87	96	8.50		11	18	156	157	9.00		14	38
283	83	90	8.48		11	18	160	163	8.99		14	29
284	80	91	8.06		12	18	157	160	8.92		13	31
	Period 2. Egg albumin ration (1.37 per cent N).											
280	86	96	7.76	107	12	34						
281	100	108	7.55	104	12	37						
282	105	114	8.50	117	9	43						
283	101	112	8.50	117	11	45						
284	100	112	8.50	117	8	42						

TABLE VII.

Summary of Biological Values.

Rat No.	Egg albumin ration.	Rat No.	Egg albumin ration.	White flour ration.	Mixed ration. Found values.	Mixed ration. Computed values.
270	83	250	87	47	64	60
271	83	251	86	46	64	59
272	76	252	84	48	66	60
273	78	253	86	46	66	59
274	84	254	87	52	67	67
Average ..	81		86	48	65	61
280	84	260	86	53	63	64
281	81	261	81	60	70	67
282	83	262	85	55	66	65
283	80	263	70	58	66	62
284	83	264	83	70	69	74
Average ..	82		81	59	67	66

effect between the nitrogen of white flour and the nitrogen of egg albumin existed than the computed values for the mixed ration would indicate. In any case, however, the supplementing effect is comparatively slight between these two sources of nitrogen, quite distinctly smaller than the supplementing relation between whole egg nitrogen and white flour nitrogen.

III. Flour and Milk.

Combinations of milk and white flour are frequent in the preparation of pastries in human dietetics. Although it is known that marked supplementary relations exist between the nitrogenous compounds of milk and those of whole cereals, we have been

TABLE VIII.
Composition of Rations.

Ingredients.	White flour ration.	Milk ration.	Low nitrogen ration.	Egg ration.
White flour	70	0	0	0
Skim milk powder	0	22.6	0	0
Sucrose	10	10	10	10
Starch	7	55.4	74	65.5–63.5
Butter fat	8	8	10	10
Cod liver oil	2	2	0	0
Salt mixture	3	0	4	4
Agar	0	2	2	2
Dried whole egg	0	0	0	8.5–10.5

unable to find any experimental results demonstrating such a relation between the nitrogen of milk and the nitrogen of patent white flour.

The composition of the rations in this experiment is given in Table VIII. The test rations were all designed to contain the usual percentage of nitrogen, namely from 1.2 to 1.3 per cent. In Period 1, a ration practically free of nitrogen was fed. In the final periods of the experiment, however, the urinary and fecal excretion on this nearly nitrogen-free ration was compared with the excretions on a ration containing a small amount of whole dried egg. The purpose of this test was to see whether, for all practical purposes, such a low egg-nitrogen ration might not be used in place of the ration containing no known source of nitro-

TABLE IX.

Nitrogen Metabolism Data.

Rat No.	Body weights. Initial.	Body weights. Final.	Food intake.	N intake.	Fecal N.	N in urine.	Body weights. Initial.	Body weights. Final.	Food intake.	N intake.	Fecal N.	N in urine.
	Period 1. Low nitrogen ration (0.022 per cent N).						Period 6. Egg ration (0.645 per cent N).					
	gm.	*gm.*	*gm.*	*mg.*	*mg.*	*mg.*	*gm.*	*gm.*	*gm.*	*mg.*	*mg.*	*mg.*
240	73	70	6.53		12	19	116	117	7.00		12	16
241	75	70	5.53		11	19	123	124	7.00		12	14
242	69	61	6.03		14	18	115	118	7.00		9	14
243	78	70	5.75		15	18	119	120	7.00		11	15
244	77	69	5.50		14	21	117	119	7.00		13	16
	Period 2. Flour ration (1.29 per cent N).						Period 7. Low nitrogen ration (0.013 per cent N).					
240	70	75	6.94	89	13	62	118	108	6.33		11	12
241	72	79	6.98	90	11	64	124	113	7.13		*	13
242	63	67	6.59	85	11	61	116	106	7.91		9*	14
243	73	79	6.69	86		62	120	110	6.54		10	11
244	72	78	6.93	89	12	66	117	108	6.49		11	13
	Period 3. Flour, milk ration (1.29 per cent N).											
240	77	90	7.00	90	12	46						
241	80	96	7.00	90	9	46						
242	69	84	7.00	90	10	45						
243	79	93	7.00	90	10	45						
244	79	93	7.00	90	11	45						
	Period 1. Low nitrogen ration (0.022 per cent N).						Period 4. Flour ration (1.29 per cent N).					
231	65	60	5.55		10	19	103	108	8.00	103	13	74
232	72	66	5.82		14	19	110	117	8.00	103	12	71
233	70	63	6.17		12	19	108	115	8.00	103	13	71
234	62	56	5.21		11	20	100	106	8.00	103	13	70
235	68	60	5.08		13	16	112	120	7.93	102	11	68

* These figures are taken from the preceding period because, for Rat 241, the fecal nitrogen determination was lost, and for Rat 242, the food intake record was unsatisfactory.

TABLE IX—*Concluded.*

Rat No.	Body weights. Initial.	Body weights. Final.	Food intake.	N intake.	Fecal N.	N in urine.	Body weights. Initial.	Body weights. Final.	Food intake.	N intake.	Fecal N.	N in urine.
	Period 2. Milk ration (1. 31 per cent N).						Period 5. Egg ration (0.747 per cent N).					
	gm.	*gm.*	*gm.*	*mg.*	*mg.*	*mg.*	*gm.*	*gm.*	*gm.*	*mg.*	*mg.*	*mg.*
231	69	86	8.00	105	13	38	111	121	8.00		14	14
232	74	89	8.00	105	14	40	117	126	8.00		12	17
233	73	91	8.00	105	14	35	118	128	8.00		14	13
234	64	83	8.00	105	12	35	110	119	8.00		12	17
235	66	86	8.00	105	15	36	121	129	8.00		11	15
	Period 3. Milk, flour ration (1.29 per cent N).						Period 6. Low nitrogen ration (0.013 per cent N).					
231	87	100	8.00	103	12	51	118	109	7.47		12	17
232	93	105	8.00	103	13	51	125	114	7.70		12	18
233	92	104	8.00	103	14	49	126	117	7.25		10	18
234	85	97	8.00	103	12	50	116	107	7.20		11	18
235	89	103	8.00	103	9	53	127	119†	7.50		9	15

† This was a 6 day period.

TABLE X.

Summary of Biological Values.

Rat No.	Milk ration.	Flour ration.	Mixed ration.	Computed for mixed ration.
231	84	50	72	61
232	81	54	72	63
233	86	53	75	64
234	88	56	75	67
235	83	55	69	64
Average. . . .	84	54	73	64
240		50	68	
241		49	69	
242		48	69	
243		47	70	
244		48	71	
Average		48	69	

gen. This substitution was considered advisable, since such an egg ration would be consumed much more readily than the low nitrogen ration.

The data of the nitrogen metabolism periods in this test are given in Table IX. For Rats 231 to 235, inclusive, the experiment was highly successful in every respect. For Rats 240 to 244, inclusive, however, in Period 4 in which the milk ration was fed, such an inadequate consumption of food resulted, leading to abnormally low biological values for the milk nitrogen, that the milk periods have not been reported with these rats. Even in a second period of milk feeding, the food consumption was not what it should have been, and abnormal biological values still were obtained. With these rats, therefore, the results of Periods 4 and 5 have been omitted from the table.

A summary of the biological values computed from the metabolism data is given in Table X. For the first group of rats, milk nitrogen possessed an average biological value of 84, practically identical with that previously reported (4). In view of the abnormally low values obtained with Rats 240 to 244, inclusive, for milk nitrogen, another group of rats was given a low egg-nitrogen ration immediately followed by a milk ration similar to the one used in the present experiment. The biological values calculated for these rats were 87, 87, 79, 88, and 86, averaging 85. It is not considered necessary to report the detailed nitrogen balance data on this test.

The biological values for white flour nitrogen are somewhat higher for the first group of rats than for the second, the respective averages being 54 and 48. Similarly for the mixed ration, the values for the first group of rats averaged 73 and for the second group of rats, 69. The biological values to be expected for this mixed ration if no supplementary relation existed, averaging 64, are all distinctly lower for the first group of rats than the values actually found. If it may be assumed that for Rats 240 to 244, inclusive, the biological value of milk proteins was actually 84, the computed biological value for the mixture would average 60, as compared with an average value of 69 found by direct experiment.

A comparison of the period in which a low egg-nitrogen ration was fed, with the period immediately following, in which a ration

practically free of nitrogen was fed, is of interest in determining whether the former ration can legitimately be substituted for the latter in standardizing rats in these protein studies. For both groups of rats the average excretion of fecal nitrogen was closely the same in the two periods; *i.e.*, 1.61 mg. and 1.58 mg. per gm. of food. For Rats 231 to 235, the average excretion of urinary nitrogen on the egg ration was slightly lower than that on the nitrogen-free ration (12.7 mg. per 100 gm. of body weight as compared with 15.5 mg.), while for Rats 240 to 244 the reverse was true (12.7 mg. as compared with 11.1 mg.). It seems fair to conclude from this test, bearing in mind the extent of the biological variations involved, that the substitution of the egg ration for the nitrogen-free ration will give practically the same standardization of the rats.

IV. Flour and Meat.

The importance of the ordinary cuts of meat in the human diet depends to a large extent upon their high content of protein of high digestibility and superior biological value. However, it should be realized that different kinds of meat and even different cuts of the same meat may vary in this respect. The ordinary assumption that the protein in cheap cuts of meat is equal in nutritive value to the protein in expensive cuts of meat can only have reference to digestibility. Any assumption that they are equal with respect to the biological value of their nitrogen is quite unfounded.

It is a matter of common observation that different cuts of meat differ in texture and particularly in tenderness. These differences presumably depend upon differences in the proportion of connective to muscle tissue. In all probability the nitrogen of connective tissue would possess a lower biological value than the nitrogen of muscle tissue.

The extent of the chemical differences existing among the nitrogen compounds in different cuts of meat is illustrated by the following data obtained in this laboratory.[1] Two cuts of meat were obtained from the same hog carcass, one a shoulder cut (picnic) and the other a tenderloin. From inspection it was evident that the shoulder cut contained more connective tissue than the tender-

[1] These results were obtained by Mr. J. H. Longwell.

loin. Upon analysis the shoulder cut was found to contain 2.89 per cent nitrogen and the tenderloin 2.94 per cent. Of the total nitrogen, 23.5 per cent was water-soluble in the case of the shoulder cut, and 28.2 per cent in the case of the tenderloin, while of the soluble nitrogen 16.3 per cent was coagulable by heat in the case of the shoulder cut, and 21.8 per cent in the tenderloin cut.

In another investigation of the same question, two different cuts of beef from two different animals were compared. The first cut was the tenderloin muscle from a young heifer in good condition. The second cut was the lower round (heel) cut from a bull. These two cuts probably showed as great a difference in content of connective tissue as can be found. The tenderloin showed no visible connective tissue, while the round cut from the bull was

TABLE XI.

Composition of Rations.

Ingredients.	Low nitrogen ration.	Veal ration.	White flour ration.
Patent white flour			67
Dried veal		10.3	
Dextrinized starch	76	65.7	10
Sucrose	10	10	10
Butter fat	8	8	8
Cod liver oil	2	2	2
Salt mixture	4	4	3

evidently extremely fibrous. The tenderloin cut contained 3.41 per cent nitrogen, while the round cut contained 3.06 per cent. Of the total nitrogen, 23.2 per cent was water-soluble in the tenderloin cut, and 16.9 per cent in the round cut. Of the soluble nitrogen, 49.9 per cent was coagulable by heat in the tenderloin cut and 47.8 in the round cut.

The first experiment dealing with meat was concerned with the biological value of the nitrogen of veal alone and with the supplementary relation existing between veal nitrogen and white flour nitrogen. The veal was obtained at a local market and contained no visible fat. It was ground, dried, reground, and passed through a sieve. During these operations it was evident that this particular cut, the location of which in the carcass was not known, contained a good deal of connective tissue.

TABLE XII.

Nitrogen Metabolism Data.

Rat No.	Body weights. Initial.	Body weights. Final.	Food intake.	N intake.	Fecal N.	N in urine.	Body weights. Initial.	Body weights. Final.	Food intake	N intake.	Fecal N.	N in urine.
	Period 1. Low nitrogen ration (0.055 per cent N).						Period 4. Flour, veal ration (1.30 per cent N).					
	gm.	*gm.*	*gm.*	*mg.*	*mg.*	*mg.*	*gm.*	*gm.*	*gm.*	*mg.*	*mg.*	*mg.*
201	117	107	5.30		9	21	139	148	7.97	103	8	55
202	116	106	5.14		10	26	127	135	7.46	97	7	53
203	114	104	5.95		10	22	120	127	6.63	86	8	48
204	118	110	5.74		11	20	124	132	7.09	92	6	56
205	116	108	5.80		11	18	123	129	7.30	95	7	55
	Period 2. Veal ration (1.20 per cent N).						Period 5. Low nitrogen ration (0.016 per cent N).					
201	117	128	7.87	94	7	56	150	133	7.24		*	19
202	116	122	7.88	95	8	58	135	126	7.83		9	17
203	113	121	7.63	92	6	50	127	120	7.21		*	15
204	117	125	7.66	92	7	53	132	126	7.34		10	14
205	113	121	7.03	84	8	49	131	122	8.19		10	16
	Period 3. Flour ration (1.29 per cent N).											
201	130	136	7.29	94	8	70						
202	126	126	7.26	94	11	69						
203	120	119	5.93	77	8	56						
204	125	124	5.47	71	7	54						
205	123	121	6.06	78	8	61						
	Period 1. Low nitrogen ration (0.055 per cent N).						Period 4. Flour, veal ration (1.30 per cent N).					
206	129	118	5.00		8	22	136	143	7.17	93		55
207	130	123	5.98		9	19	139	141	6.85	89	7	54
208	124	116	6.01		11	18	125	133	7.06	92	7	54
209	134	125	5.85		10	26	149	157	8.00	104	8	62

TABLE XII—*Concluded.*

Rat No.	Body weights. Initial.	Body weights. Final.	Food intake.	N intake.	Fecal N.	N in urine.	Body weights. Initial.	Body weights. Final.	Food intake.	N intake.	Fecal N.	N in urine.
	Period 2. Flour ration (1.29 per cent N).						Period 5. Low nitrogen ration (0.016 per cent N).					
	gm.	*gm.*	*gm.*	*mg.*	*mg.*	*mg.*	*gm.*	*gm.*	*gm.*	*mg.*	*mg.*	*mg.*
206	118	125	6.87	89	9	64	142	134	7.53		10	16
207	124	125	7.30	94	8	65	141	132	6.96		12	17
208	113	108	4.07	52	5	43	133	129	7.79		12	13
209	125	130	7.19	93	8	70	153	143	7.76		13	22
	Period 3. Veal ration (1.20 per cent N).											
206	129	137	7.50	90	9	56						
207	135	140	8.08	97	9	58						
208	118	125	7.05	85	7	53						
209	138	147	8.14	98	8	63						

* The average value for the other three rats was assumed in these cases, since the fecal nitrogen determinations were lost.

The composition of the rations used is given in Table XI. A ration practically free of nitrogen, instead of one containing small amounts of dried egg, was used in this particular test.

The complete results of the nitrogen metabolism are given in Table XII. A summary of the biological values will be found in Table XIII. The biological values of white flour nitrogen were very similar for the two groups of rats in this test, averaging 49. The values for veal nitrogen, averaging 64 and 59, were much lower than those previously obtained for this meat. The relatively poor biological value of the nitrogen of this particular sample of veal was in all probability related to its high content of connective tissue.

The nitrogen of the mixed ration, containing two parts of white flour nitrogen to one part of veal nitrogen, possessed practically the same biological value as was obtained for veal alone, the averages being 63 and 60. The biological values to be expected if no supplementary relation existed between the two sources of nitro-

gen, are in all cases less than the values actually found, averaging 9 per cent less for one group of rats and 8 per cent less for the other.

TABLE XIII.

Summary of Biological Values.

Rat No.	Biological value of flour nitrogen.	Biological value of veal nitrogen.	Biological value of nitrogen of mixed ration.	Computed biological value of nitrogen of mixed ration.
201	48	62	67	53
202	51	65	66	56
203	52	68	64	57
204	49	63	56	54
205	44	63	61	50
Average....	49	64	63	54
206	50	60	60	53
207	50	60	60	53
208	47	56	57	50
209	50	61	64	54
Average....	49	59	60	52

TABLE XIV.

Composition of Rations.

Ingredients.	Low egg-nitrogen ration.	White flour ration.	Beef ration.
Dried egg*.....	5.65	0	0
White flour.....	0	69.94	0
Dried lean beef*.....	0	0	10.17
Corn starch.....	66.35	2.06	61.83
Salts.....	4	4	4
Butter fat.....	10	10	10
Sucrose.....	10	10	10
Cellu Flour.....	4	4	4

* Ether-extracted.

The second experiment was concerned with beef.[2] The rations used are described in Table XIV. The beef was prepared in the same way as the veal in the preceding test, except that it was

[2] The metabolism data in these experiments were secured by Miss J. R. Beadles and Mr. P. M. Baldwin.

TABLE XV.

Nitrogen Metabolism Data.

Rat No.	Body weights. Initial.	Body weights. Final.	Food intake.	N intake.	Fecal N.	N in urine.	Body weights. Initial.	Body weights. Final.	Food intake.	N intake.	Fecal N.	N in urine.
	Period 1. Low egg-nitrogen ration (0.745 per cent N).						Period 4. Beef ration (1.51 per cent N).					
	gm.	*gm.*	*gm.*	*mg.*	*mg.*	*mg.*	*gm.*	*gm.*	*gm.*	*mg.*	*mg.*	*mg.*
311	89	95	8.00		13	20	112	120	8.00	120	18	66
312	76	81	8.00		11	22	107	117	8.00	120	17	67
313	65	69	6.77		11	20	95	104	8.00	120	19	63
314	70	79	8.00		10	19	104	113	8.00	120	17	63
315	71	78	8.00		11	18	105	113	8.00	120	15	66
	Period 2. Beef, flour ration (1.41 per cent N).						Period 5. Low egg-nitrogen ration (0.745 per cent N).					
311	98	101	6.26	89	9	55	116	120	7.97		15	32
312	88	93	7.65	108	11	59	118	122	7.97		13	26
313	77	83	7.46	106	11	53	106	108	8.00		16	31
314	87	91	7.61	108	11	56	113	114	7.86		12	34
315	87	94	7.86	111	12	53	112	114	8.00		19	32
	Period 3. Flour ration (1.35 per cent N).											
311	104	104	8.00	108	27	69						
312	98	101	8.00	108	22	71						
313	86	89	8.00	108	24	68						
314	94	97	8.00	108	21	71						
315	97	96	8.00	108	25	67						
	Period 1. Low egg-nitrogen ration (0.745 per cent N).						Period 4. Beef ration (1.51 per cent N).					
321	70	74	8.00		13	21	99	105	8.00	120	16	73
322	74	79	8.00		12	19	98	107	8.00	120	21	60
323	71	78	8.00		11	18	95	102	7.02	106	18	61
324	70	75	8.00		12	21	93	104	8.00	120	19	65
325	71	75	8.00		14	20	100	106	8.00	120	16	68

TABLE XV—*Concluded.*

Rat No.	Body weights.		Food intake.	N intake.	Fecal N.	N in urine.	Body weights.		Food intake.	N intake.	Fecal N.	N in urine.
	Initial.	Final.					Initial.	Final.				
	Period 2. Flour ration (1.29 per cent N).						Period 5. Low egg-nitrogen ration (0.745 per cent N).					
	gm.	*gm.*	*gm.*	*mg.*	*mg.*	*mg.*	*gm.*	*gm.*	*gm.*	*mg.*	*mg.*	*mg.*
321	78	79	7.42	96	14	64	100	91	5.37		7	40
322	80	77	6.55	85	15	56	106	102	6.18		7	36
323	81	78	6.96	90	13	63	101	95	5.14		7	33
324	76	77	7.50	97	16	66	103	96	5.72		8	38
325	77	74	7.70	99	16	68	106	102	6.25		7	32
	Period 3. Flour, beef ration (1.41 per cent N).											
321	82	92	8.00	113	10	58						
322	82	91	6.88	97	12	50						
323	83	93	8.00	113	10	50						
324	82	90	7.59	107	11	56						
325	83	93	8.00	113	12	54						

extracted with ether after drying and grinding, since it contained considerable intramuscular fat. In this test the rats received separately not only their daily allotment of 25 mg. of yeast vitamin (Harris), but also a drop of cod liver oil.

The results of the nitrogen metabolism periods will be found in Table XV. The biological values are summarized and averaged in Table XVI. The white flour nitrogen in this test had an average biological value of 55, the two groups of rats giving very constant values in this respect. The beef nitrogen possessed an average biological value of 69 for both groups of rats, the individual biological values again being quite constant. The mixed ration, containing one part of beef nitrogen to two parts of white flour nitrogen, possessed an average biological value of 70 with one group of rats, and 76 with the other. Seven of the ten rats gave higher biological values for the mixed ration than for the beef ration itself. For both groups of rats in the beef test the estimated biological value of the mixed ration, on the assumption of

no supplementing effect, was 60, so that the supplementing effect is represented by average increases in the expected biological value of 10 and 16.

Although the biological value of beef nitrogen is less than that of whole egg nitrogen, egg albumin nitrogen, or milk nitrogen, the biological value of a mixture of two parts of flour nitrogen to one part of beef nitrogen possesses at least as high a biological value as similar mixtures of white flour and whole egg, egg albumin or milk. From the experiments reported in preceding sections of this paper, such a mixture of flour and whole egg was found to

TABLE XVI.

Summary of Biological Values.

Rat No.	White flour ration.	Beef ration.	Mixed ration.	
			Found values.	Computed values.
311	53	68	64	58
312	53	65	68	57
313	57	70	72	61
314	55	73	70	61
315	58	70	74	62
Average....	55	69	70	60
321	59	71	76	63
322	56	76	75	63
323	52	69	78	58
324	55	74	75	61
325	52	67	75	57
Average....	55	69	76	60

have nitrogen with an average biological value of 75; for flour and egg albumin, mixed in a similar fashion, the average biological value was 66; while for flour and milk the value found was 71. It appears that beef nitrogen, of all the kinds of food nitrogen tested in these experiments, has the most marked supplementing effect on the nitrogen of white flour.

In experiments on the growth of rats on rations containing different sources of nitrogen, Osborne and Mendel (5) conclude that egg or milk proteins supplement flour proteins more efficiently than do meat (beef) proteins. Their results are expressed in terms

of gain in weight per gm. of protein consumed in a period of 4 weeks. With wheat flour contributing two-thirds of the protein, and at a protein level of 10.3 per cent, the average result for wheat flour and egg powder was 1.80, for wheat flour and milk powder 1.90, and for wheat flour and meat powder 1.66, when an added source of vitamin B was included in the ration. However, the gain in weight per gm. of protein consumed is influenced by the amount of food consumed, since the greater this amount in proportion to the weight of the rat, the greater will be the amount of protein consumed above the requirements for maintenance and, therefore, available for growth. In the particular experiment under discussion, the food intake of the rats on the flour and egg, and flour and milk mixtures, was quite distinctly higher than the food intake on the flour and meat mixture. Also the variation among the four rats constituting any one of the four groups of rats on experiment was such that it is extremely doubtful that the differences between the averages possess any significance.

In connection with the beef and flour experiment, some attempt was made to investigate the accuracy of the experimental technique employed in these protein studies, and to substantiate the assumptions upon which the methods of calculation are based. Some preliminary experiments performed several years ago indicated that the rat rapidly adjusts itself to changes in the quantity or character of its nitrogen intake. In order to redetermine whether the usual period of preliminary feeding of 3 days duration was adequate to permit the establishment of equilibrium with the ration, particularly with the low egg-nitrogen ration used in the standardizing of all experimental rats, the following experiment was undertaken.

Four rats were taken from the ordinary stock ration and put upon the low egg-nitrogen ration used in this experiment. The urine was collected for each of these four rats and its nitrogen content was determined daily. The results are shown in Table XVII. For two of these rats, Nos. 3 and 4, the endogenous level of nitrogen excretion was reached on the 2nd day. The adjustment was slower with Rats 1 and 2, but on the 4th or 5th day the level can be assumed to have been attained. On the basis of these results, it was considered expedient to increase the period of preliminary feeding from 3 to 4 days. This was done in the experiment on

white flour and beef, and will be adopted as a routine procedure in all future work from this laboratory.

In the calculation of the biological value of dietary protein throughout these studies, the assumption is made that if the urinary nitrogen per 100 gm. of body weight on the standardizing ration varies from the first to the final period, the change really indicates a change in endogenous catabolism rather than an error in experimental technique. It was thought that some light could be thrown upon this question by determining the creatinine nitrogen in the composite collections of the rat urine in the initial and final periods on the standardizing ration. The method used

TABLE XVII.

Adjustment of the Urinary Excretion of Nitrogen to the Endogenous Level.

Days on egg ration.	Daily excretion of urinary nitrogen.			
	Rat 1.	Rat 2.	Rat 3.	Rat 4.
	mg.	*mg.*	*mg.*	*mg.*
1	36	38	42	40
2	22	30	20	21
3	19	24	18	18
4	18	27	20	20
5	16	20	20	20
6	17	23	22	22
7	16	21	19	19
8	15	21	19	19
9	17	22	20	18
10	16	21	22	21
11	17	21	20	20

for the determination of creatinine in the weekly composite samples of rat urine was an adaptation of Folin's well known method. As used, it gives values for total creatinine, inclusive of any creatine that might be present. The details of the method are as follows:

Triplicate samples of 20 or 25 cc. each (depending upon the quantity of creatinine present) are measured out into 250 cc. weighed Erlenmeyer flasks. To one of these portions two drops of methyl red indicator are added and it is then titrated exactly to the neutral point with 5 per cent sodium hydroxide solution. The other two portions of urine are then neutralized by adding

the same quantity of NaOH. 20 cc. of saturated picric acid solution are added to these samples, which are then made up to a volume of 150 cc. with distilled water. After the addition of two or three small pieces of porous clay plate, the solutions are boiled gently over an electric hot plate, until the volume is slightly less than 20 cc. The flasks and contents are then cooled, placed on the balance, and distilled water added until the weight of the contents is 21 gm.

In preparing the standard creatinine solution, 1 cc. of creatinine zinc chloride solution (containing 0.5 mg. of creatinine per cc.) is placed in a 100 cc. volumetric flask. 20 cc. of saturated picric acid solution and 1.5 cc. of 10 per cent NaOH are then added, and at intervals of 1 minute the same quantity of alkali is added to each of the urine solutions as prepared according to the procedure described in the preceding paragraph. After standing 10 minutes, the standard solution is made up to 100 cc., and, at intervals of 1 minute, the urine samples are transferred from the Erlenmeyer flasks into 100 cc. volumetric flasks and made up to the mark. The samples are then compared in the colorimeter with the standard creatinine solution, setting the standard at the 20 mm. mark. If the reading of the unknown is less than 13 mm. or greater than 27 mm., the determination is repeated using a smaller or a larger sample as necessary.

The results of these creatinine determinations[3] in the first and final standardizing periods are given in Table XVIII in mg. of creatinine nitrogen daily, and in percentage of creatinine nitrogen on the total urinary nitrogen. The average percentage of creatinine nitrogen in the first period was 8.55 for Rats 311 to 315, inclusive, and 8.09 for Rats 321 to 325. For Period 5 the averages were, respectively, 7.89 and 8.32. The similarity in these percentages indicates that the creatinine nitrogen excretion has varied from Period 1 to Period 5 to much the same extent as the total urinary nitrogen on the low egg-nitrogen ration. This fact may be taken to confirm the assumption that the urinary excretion on this ration, after a preliminary feeding of 4 days, constitutes a good determination of the endogenous nitrogen catabolism of the rats. For example, it will be seen from Table XV that the

[3] The creatinine determinations were made by Mr. R. L. Zimmerman.

TABLE XVIII.

Excretion of Creatinine in the Urine in Initial and Final Periods on the Low Egg-Nitrogen Ration.

Rat No.	Total urinary nitrogen.	Creatinine N.		Rat No.	Total urinary nitrogen.	Creatinine N.	
		Daily.	In per cent of total nitrogen.			Daily.	In per cent of total nitrogen.
	mg.	*mg.*			*mg.*	*mg.*	
311	20.3	1.91	9.41	321	21.4	1.65	7.70
312	21.6	1.75	8.10	322	19.3	1.59	8.22
313	19.7	1.54	7.82	323	18.4	1.50	8.16
314	18.8	1.65	8.78	324	21.5	1.71	7.96
315	18.4	1.59	8.64	325	20.3	1.71	8.42
Average.....			8.55				8.09
311	31.8	2.53	7.96	321	39.6	2.99	7.55
312	26.5	2.37	8.95	322	36.0	2.86	7.93
313	31.1	2.23	7.17	323	32.8	3.21	9.79
314	33.8	2.58	7.63	324	37.9	2.93	7.72
315	32.2	2.49	7.73	325	32.0	2.75	8.59
Average.....			7.89				8.32

TABLE XIX.

Daily Excretion of Endogenous Creatinine Nitrogen per Kilo of Body Weight.

Rat No.	Period 1.		Period 5.	
	Average weight.	Creatinine N per kilo.	Average weight.	Creatinine N per kilo.
	gm.	*mg.*	*gm.*	*mg.*
311	92	21	118	21
312	78	22	120	20
313	67	23	107	21
314	74	22	112	23
315	74	21	113	22
321	72	23	95	31
322	76	21	101	28
323	74	20	97	33
324	72	24	99	30
325	72	24	104	27

total urinary nitrogen per 100 gm. of body weight of Rats 321 to 325 on the low egg-nitrogen ration increased distinctly from Period 1 to Period 5. Does this increase really signify a more intense endogenous catabolism of nitrogen or does it simply testify to an unreliable method of standardizing the rats in this respect? The fact that the creatinine nitrogen averaged 8.09 per cent of the total urinary nitrogen in Period 1 and 8.32 per cent in Period 5 supports the belief that the former supposition is the correct one, since on this ration the creatinine in the urine is presumably all of endogenous origin.

It is interesting to note that the percentage of creatinine nitrogen in the urine of the rat under conditions of feeding such that the urinary nitrogen is all of endogenous origin, is much lower than the percentage for either humans or for pigs, namely 15 to 20 per cent.

The excretion of endogenous creatinine nitrogen in relation to the body weight of these rats in Periods 1 and 5 is given in Table XIX. In general, rats of this age and weight excrete from 20 to 30 mg. of creatinine nitrogen per kilo of body weight per day. These figures are considerably higher than those previously reported by Folin and Morris (6) for the rat, *i.e.* 9 to 16 mg., which in turn are much higher than the figures for man, *i.e.* 7 to 11 mg. The figures on the rat refer to total creatinine including, according to Folin and Morris, small and inconstant amounts of creatine.

SUMMARY AND CONCLUSIONS.

The total nitrogen content of a food, rather than its total protein content, is the better starting point in its biological evaluation as a source of nitrogenous compounds in anabolism.

The average biological value of the nitrogen of white flour obtained for the forty-nine rats in these experiments is 52.

The average biological value obtained for the nitrogen of whole egg is 94, for the nitrogen of egg albumin 83, for the nitrogen of milk 85, for the nitrogen of veal 62, and for the nitrogen of beef 69.

The nitrogenous compounds in different cuts of the same kind of meat may differ greatly in chemical characteristics, indicating in particular a different proportion of connective tissue. In all

probability the varying content of connective tissue in different cuts of meat modifies the biological value of their nitrogen, the greater the proportion of the connective tissue the smaller being the biological value.

Mixtures of flour and egg nitrogen in the proportion of 2 to 1 were found to possess an average biological value of 75. If no supplementary relation existed between these two sources of nitrogen, the value expected for the mixture would be 66, so that the difference between 66 and 75 may be taken to measure the extent of the supplementary effect.

Only a slight supplementary relation was found between egg albumin nitrogen and white flour nitrogen. The average computed value for a mixture of two parts of white flour nitrogen and one part of egg albumin nitrogen was 64, while the average value obtained by direct experiment was only 66. With several of the individual rats no supplementing effect was evident.

A mixed ration containing white flour nitrogen and milk nitrogen in the ratio of 2 to 1 gave an average biological value of 71, the value to be expected if no supplementary relation existed being 62.

The biological value of the nitrogen of a mixture of flour and veal in the proportion of two parts of flour nitrogen to one part of veal nitrogen was found to be 62, the same as that found for veal alone. The expected value in the absence of supplementing effect is 53.

A similar mixture of flour nitrogen and beef nitrogen gave an average biological value of 73, higher than that of the beef alone and 13 per cent higher than the expected value if no supplementing relation existed.

Comparing the supplementary relations between white flour and whole egg, egg albumin, milk, beef, or veal, it appears that the supplementary relation is greatest between white flour and beef and least between white flour and egg albumin. No distinct differences between the other animal foods in this respect appear to exist.

The excretion of fecal nitrogen per gm. of food consumed and of urinary nitrogen per 100 gm. of body weight by the rat is practically the same on a ration containing from 0.6 to 0.75 per cent of whole egg nitrogen as on a ration containing practically no nitro-

gen. It is, therefore, proposed to substitute this low egg-nitrogen ration for a nitrogen-free ration in the standardization of rats in these protein studies.

The rat rapidly adjusts its excretion of urinary nitrogen to a change in ration. The endogenous level of urinary excretion in a rat taken from a normal stock ration is reached in from 1 to 4 days.

The young rats used in this experiment, ranging in weight from 70 to 120 gm., excreted from 20 to 30 mg. of endogenous creatinine nitrogen per kilo of body weight per day. In periods in which the endogenous level of urinary nitrogen was attained, the creatinine nitrogen constituted from 7 to 10 per cent of the total urinary nitrogen.

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FURTHER EXPERIMENTS ON THE ALLEGED INTERCHANGEABILITY OF ARGININE AND HISTIDINE IN METABOLISM.

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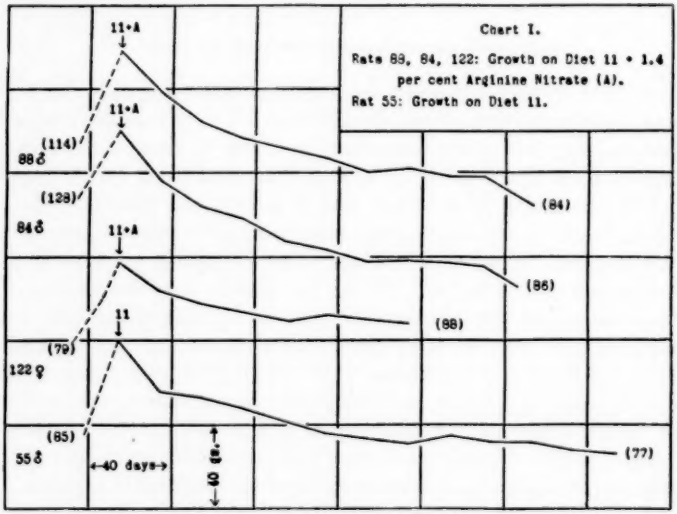
Stewart (1925) has recently reported the results of certain experiments on the relation of arginine and histidine to growth and purine metabolism. He states that while he is able to confirm the findings of Rose and Cox (1924) and of Rose and Cook (1925) concerning the importance of histidine in growth and purine synthesis, he is unable to confirm either their findings or those of Ackroyd and Hopkins (1916) regarding the effect of adding arginine to a histidine-deficient ration. It will be recalled that Ackroyd and Hopkins found the two amino acids to be interchangeable in metabolism. On the other hand, the experiments of Rose and Cox and Rose and Cook show that the addition of arginine to a diet deficient in histidine is entirely without influence upon growth or purine excretion. Animals upon such a diet continue to lose weight, and to manifest decreases in allantoin output, just as do control rats upon a similar ration without the addition of arginine.

Apparently, Stewart prefers to take a position in between the two extremes indicated above. He is of the opinion that the addition of arginine to a histidine-deficient diet inhibits the decrease in allantoin output, and renders the ration adequate for maintenance, but not for growth. Referring to his conclusion he says: "This result does not support that of Ackroyd and Hopkins who suggested that arginine and histidine are essentially equivalent—*i.e.* interchangeable—in metabolism." And again: "It is not in agreement with the observation of Rose and Cox (1924) who found that arginine, as carbonate or nitrate, entirely failed to prevent the loss in weight."

The data presented in our former papers are perfectly clear-cut and convincing. The experiments were sufficiently numerous, and extended over sufficiently long periods of time to warrant thoroughly our conclusions. On the contrary, the data of Stewart are exceedingly meager. Of the eight rats used in his investigation, four were adults employed in maintenance studies. The latter need not be discussed as they are not comparable with our experiments. The other four animals served for the growth experiments. From these he secured two growth curves, each



curve representing the combined weights of two rats. Only one of these curves shows the effect of arginine addition to the histidine-deficient ration, and that for a period of 2 weeks only. During this time, as pointed out by the author, the composite curve shows that the rats first lost weight, next gained, and then lost again. It is not surprising, therefore, that the author admits that his data are not very convincing. Indeed, one might affirm that the inconsistent results obtained in his single short experiment prove absolutely nothing concerning the interchangeability of arginine and histidine.

Recently, in connection with experiments of another sort, the results of which will be published in the near future, we have secured additional proof of the inability of arginine to supplement a histidine-deficient ration. We had not expected to publish these new data for we believed that to do so, in view of the convincing nature of our original papers, would involve useless repetition. The paper of Stewart, however, seems to render a brief presentation desirable. The experiments to which we refer are summarized

TABLE I.

Food Consumption and Body Weight Changes.

Days.	Diet.	Average daily change in body weight.	Average daily food consumption.	Days.	Diet.	Average daily change in body weight.	Average daily food consumption.
Rat 88♂.				Rat 84♂.			
		gm.	gm.			gm.	gm.
1–20	7	+2.20	9.8	1–20	7	+1.60	10.3
20–40	11 + 1.4 per cent arginine nitrate.	−1.00	5.2	20–40	11 + 1.4 per cent arginine nitrate.	−1.20	5.2
40–60		−0.70	4.7	40–60		−0.60	5.5
60–80		−0.35	3.9	60–80		−0.30	4.1
80–100		−0.25	4.2	80–100		−0.50	3.9
100–120		−0.25	4.4	100–120		−0.20	4.0
120–140		−0.30	3.7	120–140		−0.30	3.6
140–160		+0.05	5.7	140–160		+0.10	5.0
160–180		−0.20	5.2	160–180		−0.10	5.2
180–196		0	5.0	180–196		−0.13	4.9
196–212		−0.56	5.0	196–212		−0.62	4.9
212–220		−0.62	5.0				

in Chart I and Tables I and II. Each curve represents the growth of an individual and is not a composite obtained by adding or averaging the weights of two rats. The numbers in parentheses denote the initial and final weights of the animals. The part of each curve shown as a broken line represents the growth on the normal ration (Diet 7 of Rose and Cox (1924)). Three of the rats (Nos. 88, 84, and 122) were changed from Diet 7 to a ration deficient in histidine but adequate in every other respect, and containing an abundance of arginine (Diet 11 of Rose and Cox plus 1.4 per cent of

arginine nitrate[1]). Rat 55 served as a control and was transferred from the normal diet to Diet 11 without the addition of arginine. The arrows denote the points at which the dietary changes were made. The rats were kept upon the respective diets for periods ranging from 140 to 239 days. Despite the fact that the components of the rations were prepared *de novo*, and thus were not the same products used in our earlier experiments, the results of the recent studies corroborate in every respect our former findings.

TABLE II.

Food Consumption and Body Weight Change.

Days.	Diet.	Average daily change in body weight.	Average daily food consumption.	Days.	Diet.	Average daily change in body weight.	Average daily food consumption.
Rat 122 ♀.				Rat 55 ♂.			
		gm.	*gm.*			*gm.*	*gm.*
1–22	7	+1.77	7.8	1–16	7	+2.81	8.7
22–42	11 + 1.4 per cent arginine nitrate.	−0.70	3.2	16–36	11 + 1.4 per cent arginine nitrate.	−1.15	5.1
42–62		−0.30	2.8	36–56		−0.20	6.3
62–82		−0.30	2.7	56–76		−0.25	4.8
82–102		−0.20	3.1	76–96		−0.30	4.0
102–122		+0.20	4.7	96–116		−0.30	3.8
122–142		−0.10	4.1	116–136		−0.15	3.6
142–162		−0.20	3.5	136–156		−0.10	3.5
				156–176		+0.20	5.1
				176–196		−0.15	4.5
				196–216		0	5.0
				216–236		−0.20	4.6
				236–255		−0.04	4.8

The total inability of arginine to assume vicariously the functions of histidine is manifested by the immediate and prolonged decrease in weight of each animal following the dietary change. Thus it is evident that the experiments provide additional justification, if such were needed, for our former deductions.

[1] This quantity of arginine nitrate is equivalent to the sum of the arginine and histidine present in the normal diet (Diet 7).

Stewart points out that our animals lost weight less rapidly toward the end of the experiments than they did immediately following the change to the histidine-deficient diet. This is true also of the experiments herein described, but the explanation is not to be found in the addition of arginine to the ration. Exactly the same type of curve was obtained when arginine was not added to Diet 11, as is seen in the behavior of Rat 55. The growth curve of this animal is indistinguishable in character from those of the rats which received the amino acid in question. Perhaps the more rapid loss in weight during the early days of deficiency experiments may be attributed in part to the combustion of stored fat and carbohydrate, and in part to the disintegration of the less essential tissues (muscular and connective tissues) to supply the missing dietary factor for the maintenance of the more essential structures (glandular and nervous tissues). But whether this is the correct explanation or not, certainly the decrease in rate of body weight loss during the latter days of the experiments is not associated with the addition of arginine to the histidine-deficient ration.

One other matter mentioned by Stewart should be briefly considered. He states that his hydrolyzed casein, after removal of arginine and histidine by the Kossel-Kutscher procedure, responded to neither the diacetyl test for arginine nor the diazobenzene sulfonate test for histidine, and therefore could not have contained more than the merest traces of these amino acids. We are unable to confirm his findings in this regard. Although we have carried out the Kossel-Kutscher precipitation many times, and have always exercised great care to insure the presence of a large excess of silver sulfate, never have we obtained an amino acid mixture which would not respond to these tests. This was found to be true even after double precipitation with silver sulfate and barium hydroxide. Gulewitsch (1899, p. 209) has shown that the solubility of arginine-silver under the conditions of its precipitation amounts to 35 to 36 mg., expressed as free arginine, per liter of solution. Assuming that these values are correct, it is evident that the filtrate from the diamino acids, when evaporated to one-third of its original volume, must contain approximately 100 mg. of arginine per liter. Such a concentration is

sufficient to give a distinct diacetyl reaction, as may be verified by applying the test to a solution of pure arginine. When one remembers the large volume of fluid the use of which is necessitated in the Kossel-Kutscher procedure by the relative insolubility of silver sulfate, the fact is readily seen that very appreciable quantities of arginine escape removal. The diazo reaction for histidine is much more sensitive than is the diacetyl test for arginine, and would be expected to yield positive results when applied to the material in question. Our experience has repeatedly substantiated this expectation.

But the problem of complete removal is not involved in the question under discussion. We refer to it merely because in certain portions of his paper Stewart appears to have confused the question of the indispensability of arginine with the entirely different problem of the interchangeability of this amino acid and histidine. Evidently, the precipitation of histidine from our materials was sufficiently complete to render the diet inadequate for growth. It is equally certain that the addition of arginine failed to improve the quality of the food. In our former papers, as in this communication, we are concerned only with the fact that the two amino acids are not interchangeable in metabolism. We desire to emphasize again the statement made in the paper of Rose and Cox, namely, that "Our results must not be interpreted as indicating that arginine is an unnecessary component of the diet. Indeed, the data have no bearing upon this question." We are convinced that the so called arginine-histidine-free amino acid mixtures prepared by Stewart and by us contained appreciable quantities of arginine and may have carried sufficiently large amounts of this amino acid to meet the nutritive requirements of the animals. We expect to present additional information concerning this question at a later date.

The above criticism of Stewart's growth studies is applicable with equal or greater force to his investigation of the relation of arginine and histidine to purine excretion. We feel, however, that discussion of this aspect of the problem is unnecessary, for we are completely in accord with his statement that his experiment "can neither affirm nor deny that arginine has an effect on the purine excretion."

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ON THE SEPARATION OF HISTIDINE AND ARGININE.*

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(Received for publication, March 4, 1926.)

Kossel and Edlbacher (1) recently published a short paper under the above title in which they point out that a N/50 histidine solution reacts alkaline to phenolphthalein but not to thymolphthalein while a N/50 solution of arginine reacts alkaline to both indicators. They raise the question therefore, "Ist nun zur völligen Ausfällung des Histidinsilbers die Gegenwart des stärker alkalischen Arginins erforderlich, oder genügt hierzu die Entstehung von freiem Histidin?" They state that histidine is completely precipitated when the solution, containing an excess of silver nitrate, is made alkaline to phenolphthalein with barium hydroxide but remains acid to thymolphthalein; that is, when the reaction lies between the limits pH 8.3 and 9.3. An experiment is recorded in which a solution of histidine silver is boiled with barium carbonate according to the method of Weiss (2) which shows that over 99 per cent of the histidine is thereby precipitated.

In a table appended to the paper it is stated that the silver salt of histidine is completely precipitated at faint alkaline reaction to phenolphthalein but that at this reaction the precipitation of arginine silver has not commenced; furthermore when the reaction is carried over to alkalinity to thymolphthalein, arginine silver is completely precipitated. Although no experimental data are given in support of these statements, nor on their application to the separation of histidine from arginine, it is clearly implied that by the use of these indicators this separation may be effected.

* The expenses of this investigation were shared by the Connecticut Agricultural Experiment Station and the Carnegie Institution of Washington, D. C.

We therefore applied these observations during the course of an analysis of the bases from wheat gliadin. The precipitate of silver salts of the bases, obtained at a faint alkaline reaction to phenolphthalein, was found to contain considerable amounts of arginine. As this was unexpected, in view of the statements in Kossel and Edlbacher's paper, we likewise investigated a mixture of the pure amino-acids and also a solution of pure arginine carbonate. In every case a large part of the arginine was precipitated. The method, therefore, presents no advantage over that formerly proposed by Kossel and long employed in this laboratory (3).

EXPERIMENTAL.

Two lots of gliadin equivalent to 164.4 gm. of ash- and moisture-free protein were hydrolyzed, the one with hydrochloric and the other with sulfuric acid. The bases were precipitated in the usual way with phosphotungstic acid and the phosphotungstates decomposed, in the one case with sulfuric acid in the presence of amyl alcohol and ether, and in the other with barium hydroxide. To the solution of arginine, histidine, and lysine, obtained in the first experiment, an excess of silver sulfate was added, according to the original procedure of Kossel, and barium hydroxide solution added until the solution was faintly alkaline to phenolphthalein. The precipitate was removed and decomposed with hydrogen sulfide, giving a solution which contained 1.67 gm. of nitrogen. Arginine nitrogen was determined in a aliquot part by the method of Van Slyke (4) as modified by Plimmer (5) and found to be equivalent to 0.99 gm. Another aliquot part was treated with excess of Hopkins' mercuric sulfate reagent to precipitate histidine, and nitrogen determined in the precipitate. This amounted to 0.486 gm. and histidine dichloride equivalent to 0.407 gm. of nitrogen was subsequently isolated from this solution. Thus two-thirds of the nitrogen precipitated by silver under the above conditions belonged to arginine.

The solution of the bases obtained in the second experiment was treated with excess of silver nitrate and barium hydroxide added to faint alkalinity to phenolphthalein. The precipitate was removed, decomposed, and found to contain 1.715 gm. of nitrogen. Determinations, made as before, showed the presence of 0.951 gm. of arginine nitrogen and 0.285 gm. of histidine nitrogen. Histidine dichloride, equivalent to 0.204 gm. of nitrogen, was isolated from this solution.

In order further to test the precipitation of arginine under these conditions, a solution of arginine carbonate containing 1.063 gm. of nitrogen and of histidine dichloride containing 0.405 gm. of nitrogen was mixed, excess of silver nitrate added, and the solution made faintly alkaline to phenolphthalein with barium hydroxide. The precipitate was removed, decomposed, and found to contain 1.365 gm. of nitrogen. Thus nearly 93 per cent

of all the nitrogen present was precipitated. The filtrate was made strongly alkaline with barium hydroxide and the small precipitate found to contain only 0.032 gm. of nitrogen.

Finally, a solution of arginine carbonate containing 0.088 gm. of nitrogen in 100 cc. was likewise treated with excess of silver nitrate and made faintly alkaline to phenolphthalein with barium hydroxide. The precipitate, after thorough washing, contained 0.0823 gm. of nitrogen or 93.5 per cent of the arginine. A further precipitate produced by adding baryta to a strong red reaction to phenolphthalein contained 0.006 gm. of nitrogen.

Notwithstanding the failure to separate histidine from arginine under the above conditions it seems highly probable that the separation may be effected in some such manner. Kossel and Kutscher (6) long ago showed that there is a distinct break in the precipitation of the silver salts by means of barium hydroxide. Histidine comes out first completely. Further cautious addition of alkali gives no precipitate and the precipitation of arginine begins only when a "somewhat stronger excess" of barium hydroxide has been added. This behavior suggests that we are dealing with a problem in which hydrogen ion concentration plays a decisive part. It will be necessary to determine the exact point at which the precipitation of histidine silver is complete and that at which the precipitation of arginine silver begins. Whether or not this can be done and the operation controlled with sufficient accuracy by means of indicators remains to be seen.[1]

SUMMARY.

A statement in a recent paper by Kossel and Edlbacher indicates that it is possible to precipitate all of the histidine from a mixture of the silver salts of histidine and arginine, by the addition of barium hydroxide to a faint alkaline reaction to phenolphthalein, without precipitating any of the arginine. We have tested this and found that a large part of the arginine present in such a mixture is precipitated together with the histidine at this reaction. It seeems possible, however, that further investigation will show that a separation can be effected by proper control of the hydrogen ion concentration.

[1] An investigation of this point is planned in cooperation with the Sterling Chemistry Laboratory of Yale University.

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STUDIES ON THE OXYGEN-, ACID-, AND BASE-COMBINING PROPERTIES OF BLOOD.

II. A RAPID METHOD FOR THE PREPARATION OF CRYSTALLINE ISOELECTRIC HEMOGLOBIN BY THE ELECTRODIALYSIS OF RED BLOOD CELLS.

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(Received for publication, March 6, 1926.)

The methods extant for the crystallization of hemoglobin depend upon lowering the solubility of hemoglobin in its watery menstruum either (1) by changing the menstruum from water to water-alcohol or water-ether or (2) by lowering the temperature to about 0°C. simultaneously favoring the formation of relatively insoluble isoelectric oxyhemoglobin by treatment with a CO_2-O_2 mixture. The crude crystals obtained are further purified by recrystallization and finally cleared of electrolytes free or bound to the hemoglobin, by prolonged dialysis in collodion or parchment sacs.

The first method is open to the serious objection that alcohol or ether may and often does produce changes in hemoglobin (*e.g.* diminution of its oxygen-binding properties) making it unfit for many physicochemical studies.

The second method, described by Heidelberger (1923), overcomes this objection in part but not entirely. Although it is possible to obtain samples of hemoglobin with only 2 to 5 per cent "inactive" hemoglobin by this method we have usually not been able to do as well as this. On the average our samples of hemoglobin from horse blood by this method contain 10 to 15 per cent of the inactive form.

Both types of methods outlined above are laborious and as a rule at least 1 week is needed for the preparation of the crystalline product. The method given in this paper overcomes in

large measure these two undesirable factors. By the electrodialysis of washed red cells in an easily constructed apparatus the following advantages have been secured. (1) All the procedures are carried out at room temperature. (2) No foreign substance is added to the blood. (3) The short time necessary for the entire procedure (about 3 to 5 hours) eliminates practically completely the formation of inactive hemoglobin. (4) Hemolyzing agents are not needed. The cells rapidly hemolyze on account of the loss of electrolytes from them. Hence the only preprepreparation of the cells necessary is adequate washing with saline to remove serum proteins. (5) A large proportion of the inorganic ions including base bound with hemoglobin is rapidly (2 to 3 hours) dialyzed out of the hemoglobin solution. In consequence isoelectric oxyhemoglobin which is relatively insoluble in water, crystallizes out at room temperature. (6) The crystals are easily separated by centrifugation from cell debris, etc. Analysis of the crystals shows no chlorides, carbonates, or phosphates present and the base present is almost zero. A saturated solution in water at 20° shows about the conductivity of laboratory distilled water. The product is practically isoelectric hemoglobin; *i.e.*, no appreciable amounts of basic or acidic radicals are present as shown by analysis. (7) The yield is about 65 per cent of the hemoglobin originally present in the cells. (8) Determination of "total" hemoglobin and "active" hemoglobin shows that using fresh blood the crystalline hemoglobin is practically 100 per cent active.

Description of the Apparatus.

The apparatus is shown in Fig. 1 which is self-explanatory so that no more than a general description is given.

Two half boxes made of stout ($\frac{3}{4}$ inch) boards and two sheets of dialyzing parchment are fitted together with the proper interposition of rubber gaskets and held firmly by four long bolts and wing-nuts. This gives an apparatus with three separate watertight compartments; two side electrode compartments made of the two half boxes and a middle dialyzing compartment made of two sheets of dialyzing parchment. The middle parchment compartment receives the blood. All joints of the two half boxes

are mortised and filled with white lead. A covering of waterproof paint prevents warping.

The two electrodes are of carbon $\frac{3}{16}$ inch thick and are fitted into slots $\frac{1}{2}$ inch from the parchment sheets on either side. Carbon brushes for motors are easily obtainable in sizes up to $5 \times 10 \times \frac{3}{16}$ inches and when cut to proper size make convenient elec-

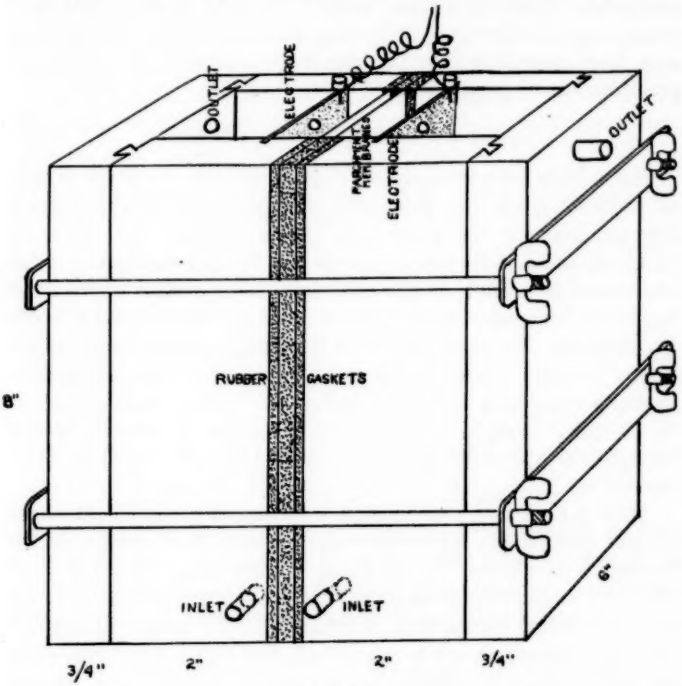

FIG. 1.

trodes. Several holes in the upper part of the electrode allow for the drainage of the water. By means of binding-posts bolted to the plates the electrodes are connected with the terminals of a 110 volt direct current.

Since considerable heat is developed during the passage of current a vigorous stream of water is kept running through each

electrode compartment. The water is introduced through two $\frac{1}{4}$ inch brass tubes opening between each electrode and its parchment membrane. Outflow occurs through two $\frac{3}{8}$ inch brass tubes placed at the top of each electrode compartment.

The rapid flow of water through each electrode compartment not only cools the apparatus but carries away products of electrode reactions. Metal electrodes cannot be used (except platinum which is prohibitive in cost) because it is difficult to prevent metal ions from migrating into the dialyzing compartment causing a precipitation of hemoglobin.

Technique of Hemoglobin Crystallization.

The middle compartment is expansible and holds 50 to 200 cc. depending on the thickness or number of rubber gaskets inserted between the parchment sheets. About 200 cc. is an optimum amount in point of time for the preparation of large quantities of crystals. The cells from 1 to 2 liters of *horse* or *dog* blood are washed three to four times in normal saline at room temperature. At least 200 cc. of the well packed cell magma are poured into the center compartment which is always adjusted in capacity by means of rubber gaskets of varying thickness so as to be full. A vigorous stream of tap water is allowed to run through the apparatus and the current (110 volt direct current) turned on.

After 3 to 4 hours, occasionally longer, there is an abundant crystal formation. The side compartments are drained and the mother liquor from the dialyzing compartment is poured off. The box is taken apart and the large mass of crystals transferred to a centrifuge tube which is centrifuged for about 10 minutes. The supernatant liquor is poured off, and the top surface of the packed crystals is washed once without mixing with ice-cold distilled water.

Preservation of Crystals of Hemoglobin.

In practice we have found it best to prepare the crystals in small batches sufficient for 1 or 2 days supply. The simplest method of preservation is to keep at ice box temperature. If kept longer than 5 or 6 days bacterial decomposition may occur. A layer of toluene over the crystals prevents this but the removal

of the toluene and the washing of the crystals once or twice with ice-cold water to remove traces of toluene entails loss of hemoglobin.

Despite any method of preservation known to us there is a slow conversion into inactive hemoglobin so that preparations cannot be preserved much longer than 1 to 2 weeks.

We have tried to obtain dried crystals by drying over sulfuric acid both in air and in nitrogen at 38°C. and 0°C. Unfortunately the dried hemoglobin so obtained, in an ideal state for preservation, has lost practically all of its oxygen capacity. We have not attempted to investigate other changes which may have occurred.

TABLE I.

Analysis of Heavily Packed Crystals of Horse Isoelectric Hemoglobin.

	Washed three times.	Not washed.
Total hemoglobin per kilo of crystals, *mM*	24.3	24.0
H_2O per kilo of crystals, *gm*	601	
Base " " " " *mM*	5	9
" mM per mM of hemoglobin	0.02	0.04
Chlorides	0	0
Phosphates	0	0
Carbonates	0	0

Composition of the Crystalline Hemoglobin.

A preparation of horse hemoglobin gave long needle crystals of hemoglobin. The results given in Table I were obtained on analysis of the crystals washed three times with ice-cold water. For comparison unwashed crystals were also analyzed.

Preparation of Hemoglobin Solutions from the Isoelectric Hemoglobin Crystals.

At room temperature a saturated solution in CO_2-free distilled water has a hemoglobin concentration of 3 mM per liter. The crystals themselves contain 40 per cent by weight of hemoglobin and 60 per cent water; *i.e.*, 40 mM of hemoglobin per kilo of H_2O. They are freely soluble in sodium hydroxide, but the maximum concentration of hemoglobin obtainable depends upon the amount of alkali added. To determine the limits of concentration of

hemoglobin obtainable with a given amount of alkali 100 gm. of crystals were mixed with 25 cc. of NaOH solution containing varying quantities of NaOH and mixed by rotation for 6 hours at room temperature. Analysis of the supernatant solution for hemoglobin and total base gave the data presented in Fig. 2 which shows as abscissæ the concentration of the hemoglobin and as ordinates the concentration of the total base in the supernatant solution. It is evident that by using sufficient NaOH it is possible to obtain a 25 mM solution of hemoglobin but with

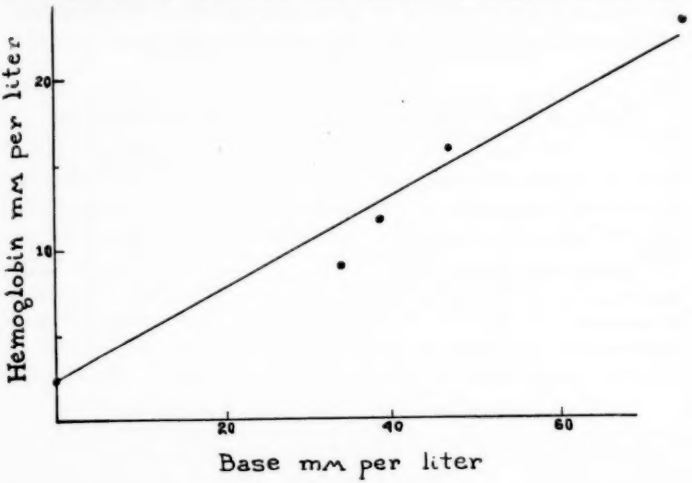

FIG. 2.

less base there is a limit to the concentration obtainable. Fig. 2 shows these approximate limits for horse hemoglobin. The type of solution used will depend upon the nature of the experimental work to be done. Any given solution within the limits shown may be prepared by adding the calculated quantity of water and NaOH directly to a roughly weighed amount of crystals, remembering that 100 gm. of crystals contain 2.4 mM of hemoglobin and 60 gm. of water. The data of Fig. 2 represent equilibrium conditions. In practice, when preparing a solution of hemoglobin rapidly the hemoglobin concentration obtained with a given amount of base may be 5 to 10 per cent lower.

Removal of Cell Detritus.

There are two alternative methods for separating the red cell ghosts and the white cells from the hemoglobin. In well washed cells these are practically the only impurities except for a small amount of cell globulins. If a small portion of the crystals obtained on dialysis of cell magma is dissolved in saline, microscopic examination of the solution shows the presence of numerous red cell ghosts of normal shape. White cells may also be seen. To obtain a hemoglobin solution free of this cell detritus it suffices to centrifuge the solution prepared from the crystals, after which the supernatant fluid may be removed for use.

The second alternative, more laborious but perhaps useful for certain purposes, is to lake the cells with ether or toluene, centrifuge, and separate the concentrated hemoglobin solution so obtained. 200 to 300 cc. of this solution which is 15 to 17 mM in hemoglobin concentration is dialyzed in the apparatus.

We have made no attempt to determine the fate of the cell globulin. Presumably most of it is removed with the mother liquor and the cell detritus.

Recrystallization.

The product obtained on the first crystallization is so pure that it suffices for practically all purposes. However, recrystallization is easily accomplished entailing, of course, a loss of about 25 to 30 per cent. A concentrated solution of hemoglobin (at least 15 mM) is prepared from the first crop of crystals and the cell detritus removed by centrifugation. 200 cc. of this solution are dialyzed and the crystals separated as before.

Preparation of Isoelectric Hemoglobin from Beef and Sheep Blood.

Isoelectric beef and sheep hemoglobins are so much more soluble than the isoelectric hemoglobin of dog or horse that it is impossible to prepare large amounts of crystals of hemoglobin from the former two species. For example an 18 mM solution of beef hemoglobin in which the base had been reduced to 5 mM per liter failed to crystallize even after 48 hours at about 5°C. However,

it is possible by procedures added to those given for horse and dog blood to prepare a concentrated solution of quite pure sheep or beef hemoglobin, with low base content, which for many purposes is eminently satisfactory.

Since no crystallization occurs the cells must be more carefully washed to get rid of the serum proteins. Four to six washings usually suffice. The cells are next hemolyzed by adding an equal volume of freshly distilled ether to the cell magma and shaking vigorously. The mixture is centrifuged and the hemoglobin solution pipetted off from the supernatant layers of ether and cell debris. 200 cc. of this concentrated hemoglobin (about 17 mM) are transferred to the center compartment of the dialyzing apparatus. If it is desired to bring the concentration of base below 10 mM per liter a slow stream of CO_2 is bubbled through

TABLE II.

Composition of Sheep, Pig, and Beef Hemoglobin after Dialysis.

	Pig.	Sheep.	Beef.
Hemoglobin, mM per liter	18	17	18
Base, mM per liter	13	11	5
" mM per mM of hemoglobin	0.7	0.7	0.3
Chlorides	0	0	0
Carbonates	0	0	0
Phosphates	0	0	0

the hemoglobin solution, which hastens markedly the dialysis of the base bound to the hemoglobin, and the electrodialysis is continued for 6 to 10 hours. If one merely desires to get rid of the chlorides and carbonates, dialysis for 3 to 4 hours suffices. The hemoglobin solution may then be tested for chlorides by igniting quickly 1 or 2 cc. in a platinum dish and testing the ash. Our usual experience has been that after 3 to 4 hours, chlorides and carbonates are completely absent and the total base varies from 10 to 20 mM per liter. While it is possible to hemolyze directly sheep and beef cells in the apparatus, we have found that hemolysis is often incomplete, so that the prehemolysis by ether, while introducing another step, is far better.

The fact that beef, sheep, and pig hemoglobins in concentrated solution do not crystallize as base is removed, while depriving us

of the advantage of crystallization as a factor in the purification of the hemoglobin, gives a certain advantage to these hemoglobins not possessed by the hemoglobins of horse or dog. A concentrated solution of these latter can only be prepared in the presence of a large quantity of base, whereas sheep, pig, or beef hemoglobin can be prepared in concentrated solution with little base. For example an 18 mM solution of beef hemoglobin may contain but 5 mM of base per liter. A similar concentration of horse hemoglobin prepared from the crystals would require a concentration of 50 mM of base per liter for solution. For certain purposes this property of sheep and beef hemoglobin is of distinct advantage. Table II gives the analysis of beef, sheep, and pig hemoglobin solutions after 3 to 7 hours of electrodialysis.

Methods of Analysis Used.

Total hemoglobin was determined by the method of Stadie (1920), "active" hemoglobin by the Van Slyke and Neill (1924) manometric oxygen capacity method, total bases by the method of Stadie and Ross (1925).

SUMMARY.

1. By means of a simple apparatus washed horse or dog red cells are subjected to electrodialysis. The cells are rapidly hemolyzed and cleared of electrolytes bound and free. A 60 to 70 per cent yield of crystalline hemoglobin is obtainable which by analysis corresponds practically to isoelectric hemoglobin.

2. A concentrated solution of hemoglobin may be prepared from sheep or beef cells which is chloride-, carbonate-, and phosphate-free, and in which the base concentration is low (0.3 mol per mol of hemoglobin).

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THE SOLUBILITY OF CALCIUM IN SERUM.

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It appears that about 20 per cent of the serum calcium is ionized (Neuhausen and Marshall, 1922), and that 60 or 70 per cent is diffusible (von Meysenbug, Pappenheimer, Zucker, and Murray, 1921). These figures show that serum calcium is not present in simple aqueous solution.

Practically all blood calcium is found in the serum (Kramer and Tisdall, 1922). The normal value for adult humans is subject to variations between 9 and 10.5 mg. per 100 ml. of serum (Kramer, Tisdall, and Howland, 1921), and the value for other animals is about the same. A decrease to 7 mg. per cent or lower is suggestive of approaching tetany in parathyroidectomized dogs (Salvesen, 1924). Hypercalcemia may also be induced in dogs by the action of parathyroid extract, with serious or fatal effects above 15 mg. per cent (Collip, Clark, and Scott, 1925). The highest values found by these authors were around 21 mg. per cent. Much higher values may be found after injection of calcium salts; and by absorption of calcium salts from relatively strong solutions introduced into the intestine values as high as 35 mg. per cent may be reached (unpublished data of the author). These examples show possibilities of considerable variation, and as the serum samples are centrifuged before determination it is probable that they represent figures for dissolved calcium. The literature does not suggest concomitant changes in the chemical characteristics of the blood sufficient to allow for such an increase in calcium solubility over the normal.

The following experiments were made to indicate the solubility of calcium in serum. As a natural characteristic subject to normal change, carbon dioxide tension was selected as the experimental variable. Calcium carbonate was selected as the solid phase because it would offer calcium ions and the carbonate ion solubility would be fixed by carbon dioxide tension.

EXPERIMENTAL.

Fresh steer serum was centrifuged after being decanted from the clot. 50 ml. were taken as the usual amount and placed with

10 gm. of precipitated calcium carbonate in a 3 liter bottle. The bottle was laid on its side and a glass tube inserted through the open narrow mouth. Through the tube gas mixtures were blown in, escaping through the open mouth. The bottle was steadily rotated by a motor-driven belt at about 30 R.P.M., spreading the serum-carbonate mixture over the walls in a thin layer. This method offered good exposure without causing frothing.

It was found that 24 hours were required to approach equilibrium conditions. As this would subject the serum to considerable evaporation if exposed to a constantly circulating gas, the gas was allowed to run in for only a few minutes in each hour, the bottle being stoppered during the remaining time.

The gases used for equilibration were (1) pure hydrogen, as used for the hydrogen electrode; (2) laboratory air; and (3) carbon dioxide-hydrogen mixtures prepared in a 35 liter tonometer.

At the end of the experiment the bottle was closed with a tubulated stopper and about 10 ml. run out under oil into a centrifuge tube. After centrifuging to throw down suspended matter samples were taken for pH and calcium determinations. Calcium was determined by Clark and Collip's (1925) modification of the Tisdall method. pH determinations were made with a De Eds-Hanzlik electrode vessel, checked against M/20 potassium hydrogen phthalate.

Carbon dioxide in the gas above the serum mixture was determined in an Orsat apparatus.

The temperature was that of the laboratory, varying between 19 and 21°.

Because of the time required for exposure of samples many different serum lots were used, each exhibiting its individual character in its normal or original calcium content and solubility. Each serum is, therefore, represented individually, and determinations were made for at least two carbon dioxide tensions.

It is conspicuous that the natural calcium content of each serum is different. Unless the carbon dioxide tension is quite high calcium is removed by mixing with calcium carbonate, so that the tension requisite for maintaining the normal calcium content is perhaps near 20 per cent. As this tension is too high to ascribe to serum in life, either the natural serum is far supersaturated or some change in its preparation reduces calcium solubility.

Centrifuging the serum may throw down with the corpuscles substances which preserve the stability of the solution. This would be easily possible in such a system as blood. Salvesen and Linder (1923) found in certain pathological cases a decrease in serum calcium parallel with the decrease in plasma protein.

TABLE I.

Calcium Concentration of Steer Serum at Various Carbon Dioxide Tensions. "Normal" Refers to Serum as Secured Clear by Decantation from the Clot and Centrifuging.

Serum No.	CO_2 tension.	Time.	Calcium.	pH	
	atmospheres	*hrs.*	*mg. per cent*		
1	Normal.		10.6		
	0		5.2	8.6*	
	0.053		10.1		
2	Normal.		11.5		
	0	3	10.6		
		12	8.8	8.94	
	0.107	7½	9.4	6.63	
		9	10.2	6.26	
	0.103	6	12.6	6.59	Calcium
	0		7.8	8.39	phosphate.
3	Normal.		11.7		
	0		6.5	9.43	
	0		6.6	9.60	
4	Normal.		11.0		
	Air.		7.0	7.71	
	0.184	20	10.7	6.48	
5	Normal.		10.3		
	0.47		12.0	6.0*	
	0.96		17.1	5.6*	
	0		5.8	8.88	

* Colorimetric determination.

Calcium bears intricate relations with the mechanism of clotting, but not in a way shown to influence its solubility. For calcium carbonate solutions a fall in temperature does not diminish but increases solubility. Serum which had stood for several days in the ice chest showed no calcium change, so the difference is not to

be ascribed to age or cooling. As precipitating influences, agitation and solid particles of calcium carbonate might be effective. However, calcium supersaturation seems to be a relatively stable condition of serum.

The single pair of observations on calcium phosphate shows a higher solubility where carbon dioxide tension equals 0.103, and a lower solubility in a hydrogen atmosphere, even though the serum did not become so alkaline as the carbonate mixture.

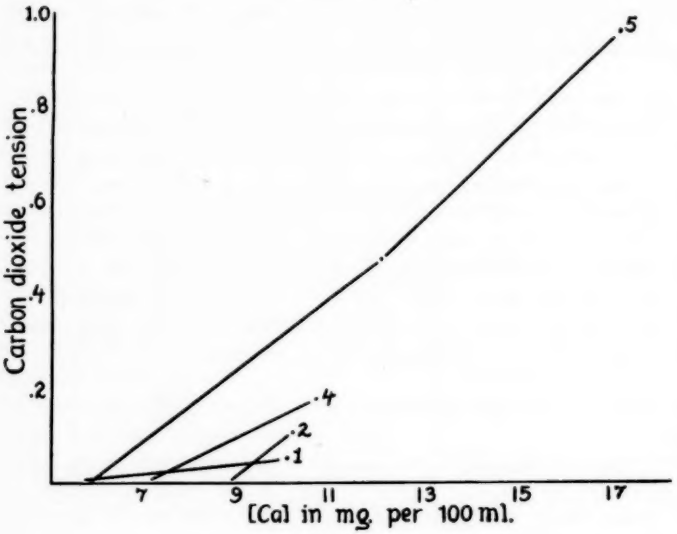

FIG. 1.

The curves representing the data show the difference in behavior of each sample of serum. In making these curves it was assumed that in the hydrogen and air experiments carbon dioxide was sufficiently near to zero to be considered as such. The check on this point of view is furnished by the pH determinations.

Calcium solubility increases with carbon dioxide tension. Plotting against pH gave less satisfactory curves than against carbon dioxide tension.

Rona and Takahashi (1913) showed that calcium carbonate

solubility varied as C_H and inversely as bicarbonate concentration. Holt, La Mer, and Chown (1925) studied the supersaturation of a calcium phosphate system comparable to blood and emphasized the time required for equilibrium. I have found calcium in sea water over a culture of a calcareous ciliate more than twice saturated, in a culture over a year old. Heubner (1923) found suspended calcium phosphate pharmacologically more active than soluble calcium injections, although it is not certain that the symptoms are purely those of calcium disturbance. The findings suggest that physical constitution of the organism or the calcium system may be determinants of calcium ion concentration in blood.

CONCLUSIONS.

1. Serum precipitates calcium when in contact with calcium carbonate unless the carbon dioxide tension is about 0.2 atmosphere.

2. Solubility varies with carbon dioxide tension, but not rapidly nor at the same rate for different serum samples.

3. The insensitivity of the system to carbon dioxide tension suggests that it is not an important vital character in determining calcium concentration of serum.

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THE TITRATION OF ORGANIC ACIDS IN URINE.

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The simple titration method for the determination of organic acids in urine, described by Van Slyke and Palmer (1), has in many circumstances proved satisfactory. A more extended employment of the method to determine organic acids in pathological urines has brought to light limitations and sources of error which need consideration.

The organic acids present in urine, both free and as salts, are estimated by titration, using suitable indicators, between the hydrogen ion concentrations represented by pH 8 and pH 2.7 respectively, after removal of the phosphates and carbonates by means of calcium hydroxide. By this procedure approximately 95 per cent of the organic acids may be determined. Weak bases, such as creatinine, creatine, and amino acids are included in the titration. A method of estimating the influence of these substances is described in the original paper.

Precipitation of Phosphates and Carbonates by Calcium Hydroxide.

Carbonates.—While in normal and most pathological urines an excess of $Ca(OH)_2$ takes out all the carbonates, it has been found that when the carbonates, calculated as sodium bicarbonate, are present in the urine in amounts greater than $\frac{1}{2}$ per cent they are not completely removed. At least this is true unless the sample with the $Ca(OH)_2$ be allowed to stand for several hours with frequent shaking, or is brought to the boiling point. If carbonates are present in appreciable amounts we prefer to add hydrochloric acid, 10 per cent, and shake the sample vigorously until most of the CO_2 thus liberated is driven off. Caution in this respect is therefore necessary whenever alkalies are admin-

istered. In any urine with a pH greater than 7 the calcium hydroxide filtrate should be examined for the presence of carbonates. It is evident from recent publications (2, 3, 4) that the possibility of inadequate precipitation of the carbonates by calcium hydroxide is not generally appreciated.

Phosphates.—Less difficulty has been encountered in removing the phosphates with $Ca(OH)_2$. The phosphate content of urine calculated as P_2O_5 is seldom over 0.3 per cent. In our experience the phosphates are completely removed in any concentration up to 0.35 per cent. To insure complete precipitation, therefore, a specimen with amounts greater than this should be diluted one-half.

Albumin.—Complete removal of albumin from the urine is essential. Small amounts of albumin are taken out by calcium hydroxide, but there seems to be considerable variability in this respect. It is advisable to test the filtrate for albumin in every urine specimen which contains albumin before precipitation. Adding one to three drops of concentrated HCl to the 100 cc. of urine, bringing to the boiling point, and filtering, removes most of the protein.

Indicators.

Errors in the use of indicators for the determination of hydrogen ion concentration of biological solutions have been frequently emphasized (5, 6, 7). The chief difficulties arise from the presence of proteins and salts. There is great variation among the indicators in their behavior under varying conditions. Aside from the possible effect on the actual hydrogen ion concentration of the solution, protein may interfere in several ways. A combination between protein and indicator may take place such that the color of the unknown is entirely changed, rendering any comparison with a standard impossible. Varying amounts of the indicator may be absorbed by the protein, thereby reducing the effective concentration of the indicator. A third difficulty arising with a few indicators is an apparent precipitation of the dye resulting in the same effect for practical purposes as absorption. Furthermore, protein takes part in the titration.

Within the limits in which the procedure is recommended, the error due to the presence of salt is probably not great enough to diminish the usefulness of the method. Carbonates and phos-

phates are removed, the chlorides are the important salts remaining. Unless the chlorides are present as ammonium salts they apparently do not affect the titration values greatly. The widest variation we have found is as follows: 25 cc. of urine with a chloride concentration (calculated as sodium chloride) of 0.26 per cent titrated with 0.2 N acid, 11.2 cc.; with 1.0 per cent, 10.8 cc.; with 2 per cent, 10.6 cc.; with 3 per cent, 10.4 cc.; producing a maximum effect of 0.8 cc. of 0.2 N acid or 64 cc. of 0.1 N acid per liter. Although it may be possible to determine the "salt effect" correction more accurately the limitations of the method and the practical use for which it is designed do not seem to warrant such a course. The effect of ammonium salts is discussed in the original communication.

Sulfates are present in the urine in much smaller amounts than chlorides and the error due to their presence does not appear significant.

Tropeolin OO.—As originally advised tropeolin OO has proved to be the more convenient indicator. It is only slightly affected by the urinary pigments and changes rapidly to a deeper red at the end-point (pH 2.7) used. After a little experience the comparator box may be omitted. Certain difficulties, however, have been encountered. One of the most disturbing factors has been fading as the end of the titration is reached. Great variability in this respect is experienced. Occasionally no fading is noticed until 15 minutes to an hour after the titration has been completed. On the other hand, fading may occur almost instantaneously rendering an estimation impossible. That this is due to a particular effect on the indicator by some unknown substance in the urine seems probable, since fading does not occur when other indicators are used. In some of the specimens when fading has occurred, if the tube is allowed to stand exposed to the air the upper part of the solution turns red, suggesting that the interference is due to some easily oxidizable substance in the urine. Diluting the urine one-half or allowing it to stand 24 hours in some instances causes the fading phenomenon to disappear. More frequently, however, these procedures are of no avail. We have eliminated the ingestion of drugs as being responsible for the difficulty. Aluminum hydroxide, charcoal, fullers' earth, copper hydroxide, lead hydroxide, and colloidal iron have been disap-

pointing in circumventing the trouble. The addition of iodine to a slight excess, allowing to stand 10 minutes, and decolorizing with sodium thiosulfate solution will generally abolish or diminish the fading phenomenon, but unfortunately this procedure affects the titration and serves no useful purpose. When fading occurs with tropeolin OO another indicator must be used. Bromophenol blue is the one we prefer.

A wise precaution in the titration with tropeolin OO is to allow the sample to stand 15 minutes after the end-point is reached and again compare with the standard in order to determine the presence of fading.

Bromophenol Blue (*Tetrabromophenolsulfonephthalein, Clark and Lubs*).—Next to tropeolin OO, bromophenol blue has been the most satisfactory indicator. The chief objection is that at the pH 2.7 the color change is so gradual the end-point is not sharp, also the greenish yellow color is somewhat influenced by the urinary pigment. The comparator box aids greatly in the determination of the end-point. Fading has never been noted.

Methyl Orange.—The character of the color is so changed by the urinary pigment that the end-point is unsatisfactory with or without a comparator box. Attempts by well known methods (charcoal, aluminum cream, kaolin, fullers' earth, and colloidal iron) to remove or reduce the urinary pigments have been disappointing.

Dimethyl Aminoazobenzene.—The difficulties in the use of dimethyl aminoazobenzene are similar to those encountered in the use of methyl orange. Also the standard fades rapidly and must be renewed frequently.

CONCLUSIONS.

1. In the use of the method for the titration of organic acids in urine as described by Van Slyke and Palmer all protein, carbonates, and phosphates must be removed.

2. While tropeolin OO is the most satisfactory indicator for general use, occasionally urine specimens contain some unknown substance which produces fading as the end-point is reached. Such specimens should be checked using another indicator, preferably bromophenol blue.

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STUDIES ON THE STABILITY OF EVAPORATED MILK DURING STERILIZATION, WITH REFERENCE TO THE HYDROGEN ION CONCENTRATION, ALCOHOL TEST, AND THE ADDITION OF SPECIFIC BUFFERS.

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The stability of protein solutions when heated presents one of the most intricate problems in colloid chemistry, particularly in biological fluids where conditions are complicated by the presence of buffer salts, carbohydrates, varying degrees of acidity, and other little known and difficultly controlled factors. In the manufacture of evaporated milk, which must be sterilized by heat, problems arise which are important industrially and also are of interest to students of colloid systems in general. The practical object of research along this line is twofold—information as to possible methods of increasing the stability of evaporated milks which might prove troublesome in the sterilizer, and the development of tests by which unstable milks can be recognized.

Experience has shown that measurements of the coagulation time of fresh milk cannot be relied upon to indicate the behavior of the evaporated product on being sterilized. That the acidity of milk greatly influences its coagulation by heat is a matter of general knowledge, but routine neutralization of excessive acidity by bicarbonate or other methods does not by any means solve the problem. There is considerable evidence that the proportions of certain anions and cations present are of great importance, and a variety of observations on these points may be found in the literature, many of which, however, are contradictory. The behavior of a milk when mixed with an equal quantity of alcohol has been

used as a criterion of its stability. Those milks which show a perceptible precipitate when so treated are called alcohol-positive. Various strengths of alcohol have been recommended, particularly 65, 70, and 75 per cent.

The data here presented were collected during a study of factors influencing the stability of evaporated milk in the sterilizer, particularly hydrogen ion concentration and the effect of the addition of certain anions, as well as the question of a possible relationship between the alcohol test of the original milk and the stability of the finished product to sterilization. The milk used in these tests was probably superior to that encountered in factory practice. It was produced at the United States Department of Agriculture experimental farm at Beltsville, and was received at the laboratory about 5 hours after milking. The cows were on controlled rations, and during practically all the work reported, were not pastured. Late in January, 1925, it was noticed that the mixed herd milk was alcohol-positive, though of excellent quality according to ordinary tests and low in bacteria count. Tests made at milking showed that 41 out of 54 cows tested gave alcohol-positive milk. Careful study of the farm records excludes the factors usually offered as explanation of milk secreted positive, such as age of cow, stage of lactation, inflammation of the udder, change of feed, etc., as no correlations could be found. Some of the cows whose milk was positive were fed silage, others were not; and the same was true of those whose milk was negative. This state of affairs afforded an opportunity to study the relationship between the alcohol test and heat stability and the possibilities of improving the stability of such milk by artificial means.

In the hope of obtaining milk of uniform quality, so that observations on different days would be comparable, it was decided to make a study of the milk of a single cow. The one chosen was a grade Holstein, 6 years old, in the 2nd month of her third lactation. Her ration during the month of February was: grain, 318 pounds; hay, 318 pounds; and silage, 1008 pounds. She yielded 1208 pounds of milk during the month, with 47.1 pounds of milk fat. Her milk was definitely positive to 75 per cent and to 70 per cent alcohol throughout this period, though toward the end the precipitate with 70 per cent alcohol was less marked than at first.

On 20 different days in February, the stability at sterilization temperatures and the hydrogen ion concentration were determined, and attempts were made to improve the stability by addition of buffer solutions, noting carefully any changes in pH or alcohol reaction. All the buffer solutions used were $\frac{1}{20}$ molal in respect to the anion, the pH being regulated by the amount of NaOH added to the acid when making up the solution. By using two or more buffers with different percentages of neutralization, samples of milk could be prepared containing equal amounts of anion but of different hydrogen ion concentration. To determine stability the samples were forewarmed at 95°C. for 10 minutes, evaporated at about 50°C. in partial vacuum to 18 per cent solids not fat, sealed in 6 ounce cans, and sterilized at 120°C. The stability is recorded as minutes of sterilization at 120°C., which is necessary to curdle the product. The probable experimental error is about 1 minute. In this manner results can be obtained which are comparable with actual observations in factory practice. Hydrogen ion concentrations were determined electrometrically.

As a matter of fact, the milk from this cow was not uniform from day to day, the total solids varying from 11.49 to 12.93, the pH from 6.48 to 6.60, and the coagulation time from 4 to 17 minutes, with the exception of two samples near the end of the month which held up for 20 and 24 minutes respectively. The average coagulation time, including these last two, was 12 minutes. Table I gives the essential data in this series of experiments. Borates were used at first, as borate lies near citrate in the Hofmeister series, and exhibits similar peptizing properties without itself upsetting the calcium-magnesium-citrate-phosphate balance. On later samples, the effect of citrate was compared with that of borate.

The most noticeable fact which appears is that whether citrate or borate is used in a milk susceptible of improvement, discretion must be used in choosing the quantity added, as increased amounts of borate or citrate can at best increase stability only up to a certain point, beyond which the stability is decreased. There was evidently no particular pH to which the milk could be arbitrarily adjusted with uniform results; each sample seemed to have its own optimum in this regard, although natural or adjusted samples lying above or below the pH range 6.55 to 6.65 were never

TABLE I.

Variations in the Original Condition of the Milk of a Single Cow during a Month; Results of Treatment with Buffer Mixtures.

Date.	Original milk.				Treatment.			Treated milk.		
	Total solids.	pH	Alcohol test.	Coagulation time.	Anion.	pH of solution.	Amount per 100 cc. of milk.	pH	Alcohol test.	Coagulation time.
Feb.				*min.*			*cc.*			*min.*
3	12 04	6.50	+	7	Borate.	7.94	6.0	6.58	±	27
	12.04	6.50	+	7	"	10.56	6.0	6.81	−	0*
4	12.19	6.48	+	11	"	10.56	3.75	6.65	−	11
	12.19	6.48	+	11	"	10.56	6.0	6.73	−	5
5	12.34	6.51	+	8	"	10.56	3.0	6.66	−	10
	12.34	6.51	+	8	"	10.56	6.0	6.82	−	0*
6	11.95	6.56	+	10	"	10.56	2.25	6.63	−	21
	11.95	6.56	+	10	"	10.56	4.0	6.81	−	3
9	12.06	6.50	+	8	"	10.56	2.2	6.63	±	26
	12.06	6.50	+	8	"	10.56	4.0	6.72	−	3
10	11.83	6.50	+	6	"	10.56	1.6	6.60	±	29
	11.83	6.50	+	6	"	10.56	2.1	6.62	−	20
11	12.53	6.53	+	10	"	10.56	1.0	6.60	−	20
	12.53	6.53	+	10	"	10.56	2.0	6 64	−	4
	12.53	6.53	+	10	"	10.56	3.0	6.71	−	1
12	11.49	6.52	+	14	"	10.56	2.0	6.63	−	14
	11.49	6.52	+	14	"	10.56	2.4	?	−	5
	11.49	6.52	+	14	"	10.56	2.8	?	−	4
13	12.80	6.54	+	8	"	10.56	2.0	6.65	±	18
	12.80	6.54	+	8	"	10.56	2.25	6.67	−	13
14	11.76	6.51	+	8	"	10.56	2.0	6.66	−	14
17	12.60	6.51	+	8	"	10.56	1.8	6.62	+	5
	12.60	6.51	+	8	"	10.56	2.0	6.63	+	4

* Coagulated before sterilization temperature was attained.

TABLE I—*Concluded.*

Date.	Original milk.				Treatment.			Treated milk.		
	Total solids.	pH	Alcohol test.	Coagulation time.	Anion.	pH of solution.	Amount per 100 cc. of milk.	pH	Alcohol test.	Coagulation time.
Feb.				*min.*			*cc.*			*min.*
18	12.93	6.54	+	10	Borate.	11.95	1.5	6.60	±	17
	12.93	6.54	+	10	"	11.50	1.5	6.60	+	16
	12.93	6.54	+	10	Citrate.	11.66	1.0	6.55	+	16
19	12.70	6.56	+	13	"	11.66	2.0	6.58	±	22
	12.70	6.56	+	13	Borate.	11.95	1.35	6.61	±	17
	12.70	6.56	+	13	"	11.50	1.4	6.61	±	20
20	12.64	6.55	+	20	"	11.95	1.0	6.59	±	19
	12.64	6.55	+	20	Citrate.	11.66	2.0	6.58	±	22
21	12.15	6.56	+	17	"	11.66	0.8	6.55	±	32
	12.15	6.56	+	17	Borate.	11.95	1.0	6.58	±	26
24	12.32	6.55	+	15	Citrate.	11.66	0.5	6.55	±	20
	12.32	6.55	+	15	Borate.	11.95	0.8	6.57	±	26
25	11.98	6.60	±	24	Citrate.	11.66	0.4	6.60	±	20
	11.98	6.60	±	24	Borate.	11.95	0.6	6.60	±	26
26	12.69	6.52	+	16	Citrate.	11.66	0.9	6.52	+	15
	12.69	6.52	+	16	"	11.66	1.1	6.53	+	16
	12.69	6.52	+	16	"	11.66	1.3	6.55	±	22
27	12.61	6.57	+	15	"	11.66	1.0	6.60	+	20
	12.61	6.57	+	15	"	11.66	1.3	6.59	−	18
	12.61	6.57	+	15	"	11.66	1.6	6.61	−	24
28	12.2	6.57	+	10	"	11.66	3.5	6.58	±	29

stable. The alcohol test afforded a much better means of avoiding overneutralization. Whenever the milk received enough buffer to render it decidedly alcohol-negative, the point of maximum stability had been exceeded, and the milk might be even less stable than before treatment. Satisfactory results could be

obtained only when the milk was rendered faintly negative; that is, when there was a barely perceptible precipitate with equal quantities of milk and 75 per cent alcohol and none with 70 per cent alcohol. This point is indicated in the data as "Alcohol ±." Similar observations were made on the milk from other cows in the herd.

This study indicated that mere treatment of a milk by citrate or borate could cause no definitely predictable change in stability, unless other factors were controlled, and that unless a considerable series of determinations was made on each sample, discordant if not contradictory findings might be expected. As no cow gave a sufficient yield at any one milking to furnish samples for an extended series, the rest of the work on alcohol-positive milk was done on mixed samples from four cows, two Jerseys and two Holsteins, which consistently gave a positive alcohol test. Many series were studied of which only a few typical examples can be shown here. As the daily samples show considerable variation, both in original condition and response to adjustment with buffers, anything in the nature of a statistical summary would involve averages of results due to changes in unlike and perhaps unrelated variables, and would of necessity be misleading.

Chart I is a typical curve illustrating the effect of adding citrate buffers to an alcohol-positive milk. Amounts of citrate added, expressed as cc. of twentieth molal citrate per 100 cc. of milk, are indicated on the x axis; stability is indicated on the y axis in terms of minutes at 120°C. to cause coagulation. To avoid the use of complicated three dimensional diagrams, the pH is indicated at each sampling point, and beneath it the result of the alcohol test (+, −, or ±). The continuous line shows the effect of the addition of a citrate-NaOH mixture having a pH of 10.17; the dotted line records the results obtained with a mixture much more acid than the milk itself (pH = 3.76). As both solutions were $\frac{1}{20}$ molal citrate, the specific effect of citrate, whatever it may be, should be the same on any two samples of milk containing equal amounts of citrate. The great difference in stability in the two samples treated with 2 cc. of citrate must obviously be due largely to differences in pH. The one at 6.64 held up for 19 minutes after the sterilizer came to temperature, that at 6.50 was curdled in 3 minutes. It should be most particularly noted, however, that the

mere addition of a citrate much less acid than the milk could not be relied upon to produce improvement, as the sample receiving 3 cc. of the citrate mixture at pH 10.17 is hardly better than the original milk. Evidently we have a delicate balance between a citrate effect and a pH effect. Maximum stability depends on optimum adjustment of these two factors. Since throughout the

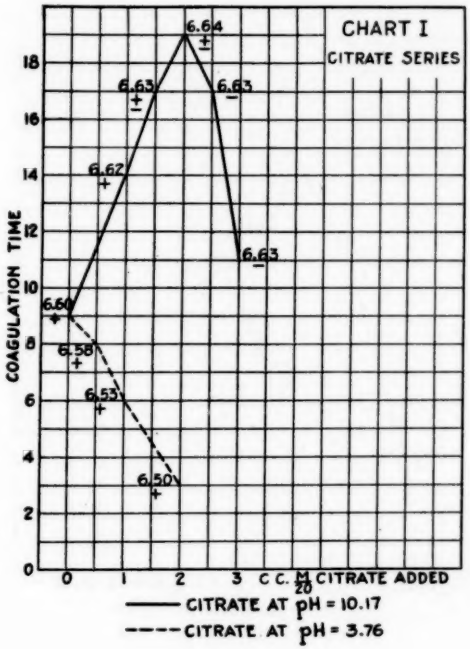

Chart I.

upper curve there is no change in pH which can be detected in milk except by electrometric methods, the question arises, is there any means of recognizing this optimum point, beyond which further treatment works disaster?

The alcohol test has been advocated by some and discarded by others. The relationship between heat coagulability and alcohol coagulability is not known. That they are not the same thing,

nor due to precisely the same mechanism, is obvious from a wealth of experience by many investigators. Neutralizing the acid or readjusting the buffer balance so that an alcohol-positive milk becomes negative does not necessarily increase its stability toward heat. On the contrary, we have often observed alcohol-negative milks whose heat resistance became greater on slight increases of

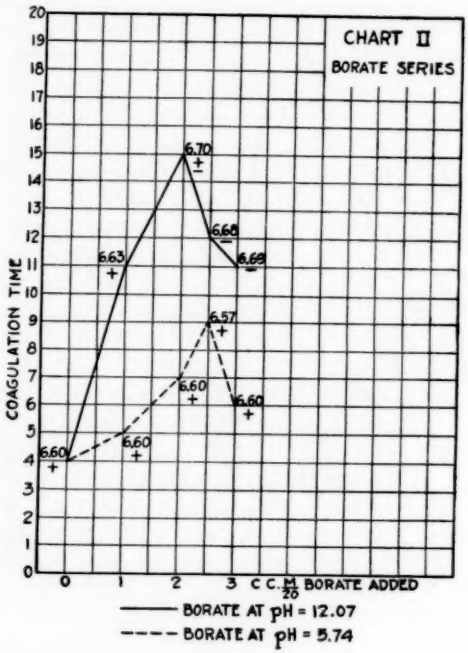

Chart II.

acidity. In all the curves here shown, and in almost all of others too numerous to present here, the maximum stability occurred at the ± point. This was true of relatively unstable milks of both the alcohol-positive and the alcohol-negative types.

Chart II shows a similar experiment with borates, which is even more significant, as one buffer was so adjusted that it would not appreciably change the pH of the sample. To accomplish this,

the buffer must be somewhat more acid than the milk. The dotted line shows the effect of addition of borate without much change in pH. This gave a maximum resistance of 9 minutes when 100 cc. of milk were treated with 2½ cc. of a borate solution whose pH was 5.74 and a drop back to 6 minutes with more borate. A borate mixture at pH 12.07 neutralized some of the slightly

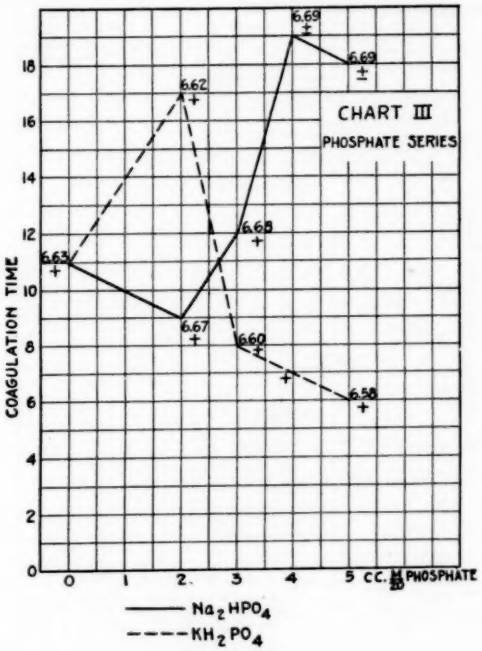

CHART III.

excessive true acidity in this particular milk with marked improvement, since 2 cc. of this solution enabled the milk to hold up for 15 minutes. At the pH thus produced, however, further addition of borate cannot be borne, and causes rapid decrease in coagulation time. It seems highly probable that with a buffer which would introduce 2½ cc. of borate and at the same time leave the pH considerably below 6.70, an even better result could have been

obtained, as our findings in general indicate that the optimum range of pH for milks from this herd is between 6.58 and 6.65.

When phosphates were used, the curves showed unexpected peaks and breaks, whether the adjustment was toward the acid or the alkaline side. One of these (Chart III) is included to point out graphically the contradictory or misleading results which might be

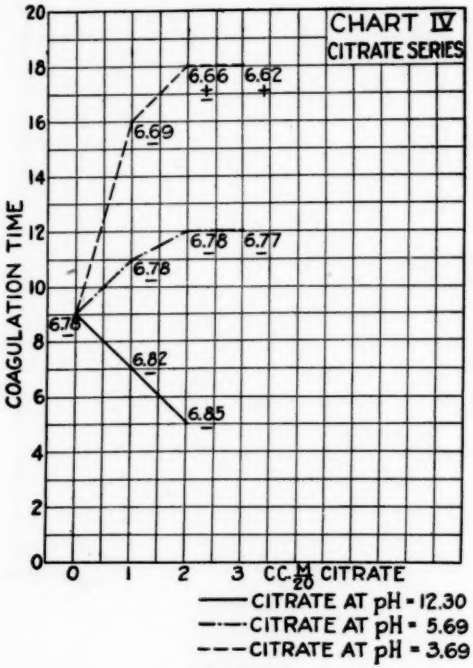

Chart IV.

obtained in attempting to study the effect of phosphate on stability, unless a fairly long series is studied for each sample. It should also be noted that there were days when treatment with citrates or other buffers reduced the coagulation time instead of increasing it. Some of these milks were fairly resistant in their original state, holding up for 18 or 20 minutes, and were perhaps secreted in a state of excellent balance in respect to heat resistance.

Other samples, particularly in the early stages of the work, unfortunately were treated with buffers so alkaline that the milk was overneutralized and the pH effect overshadowed any increased stability due to the anions added.

It seemed advisable to study the effect of similar readjustments on alcohol-negative milk. A series of mixed samples from four normal cows, two Jerseys and two Holsteins, was used. These

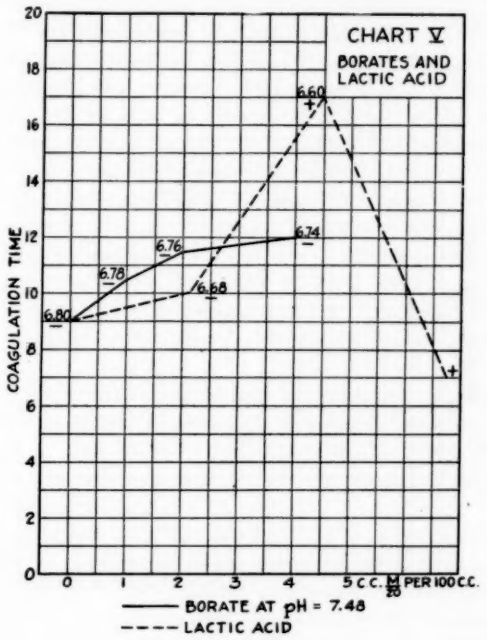

CHART V.

mixed samples ran fairly uniform during May, the solids not fat varying from 8.47 to 8.97, the pH from 6.72 to 6.82, and the coagulation time from 7 to 13 minutes. This is a type of milk which appears excellent in quality, but may prove troublesome in the sterilizer. Chart IV shows the effect of treatment with various amounts of three different citrate solutions, all $\frac{1}{20}$ molal in citrate, but so adjusted that one depressed the pH (dotted line), one raised

it (solid line), and one did not change the pH, thus indicating the effect of citrate apart from adjustment of true acidity (dots and dashes). Increase of stability is manifest up to addition of 2 cc. per 100 cc. of milk, but this effect is greatly enhanced by using an acid citrate mixture, and thus depressing the pH to a value from 6.66 to 6.62.

Chart V shows a similar slight improvement when borates were

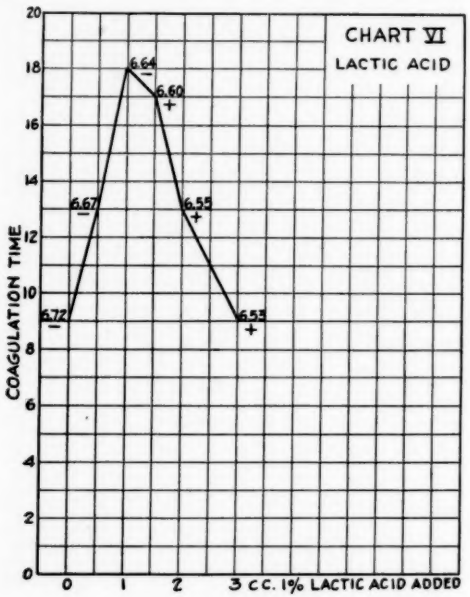

Chart VI.

used in an alcohol-negative milk, though here the improvement is probably due in part to change of pH as well as to the borate. 1 per cent lactic acid sufficient to bring this sample to pH 6.60 worked a marked improvement in stability. (This is reduced for purposes of plotting to terms of twentieth molality of lactate, but the actual volumes added were 1, 2, and 3 cc. respectively.) Chart VI gives the curve obtained when 1 per cent lactic acid (about $\frac{1}{9}$ molal) was added to an alcohol-negative mixed milk of

pH 6.72. Here maximum stability was attained at pH 6.64, just before the alcohol test turned positive.

SUMMARY.

From the experiments recorded here, and from other data which space does not permit to be presented, the following conclusions appear to be justified:

1. The milk of individual cows varies considerably from day to day in pH and resistance to heat coagulation. Consequently each sample, whether mixed or from a single cow, must be regarded as a separate colloidal system. Such experimental controls as are necessary must be run on the sample in question, as results obtained from any other sample, even though from the same source at a subsequent milking, cannot be assumed to be comparable.

2. Stability under sterilization is considerably affected by addition of citrates. This cannot be due entirely to a specific effect of citrate itself, but rather to its buffer action and peptizing effect, since similar results can be obtained with borates. In a milk which can be stabilized in this manner, the maximum increase in stability is easily overreached, and overtreatment may reverse the effect.

3. The pH effect may completely overshadow any action of the buffer solutions studied, especially if the sample lies above or below a pH range of about 6.58 to 6.65. Within this range changes in salt balance are very important; outside it, changes in pH produce more marked effect.

4. The mere fact that the alcohol test is positive or negative does not prove anything as to the heat stability of milk. Turning an alcohol-positive milk negative by citrates or other buffers does not increase the stability unless the optimum combination of pH and salt balance is approached. This optimum is probably the resultant of several variables and consequently is an expression of the colloidal peculiarities of the particular sample. It is different in different milks. In the majority of the samples studied, however, the optimum lies at or near the point where the milk is positive to 75 per cent alcohol and negative to 70 per cent.

HYDROLYSIS OF SUCROSE BY INVERTASE IN THE PRESENCE OF α-METHYL GLUCOSIDE. II.*

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(Received for publication, March 23, 1926.)

It has been pointed out in previous papers from this laboratory that the hydrolysis of a 10 per cent sucrose solution by invertase, obtained from a brewery yeast, can be represented by the empirical equation (1)

$$N = \frac{1}{t}\left[\log \frac{100}{100 - p} + 0.002642\, p - 0.00000886\, p^2 - 0.0000001034\, p^3\right] \quad (1)$$

in which N remains constant throughout any one hydrolysis (if the latter is not deviated from its normal course by the inactivation of the enzyme or for some other reason) (see Table I A). The quantities p and t represent the per cent sucrose hydrolyzed and time respectively.

Presence of Added Invert Sugar Modifies the Course of Hydrolysis.—When invert sugar is added to the 10 per cent sucrose solution the hydrolysis is retarded. By applying equation (1) to the data obtained from such a hydrolysis, Table I B, it will be noticed that the values for the parameter N are no longer constant but decrease as the reaction proceeds. Since N may be looked upon as a measure of the velocity of hydrolysis, this decrease shows that not only is the reaction slowed up as if less invertase had been used, but the rate becomes less and less as the hydrolysis progresses.

Influence of Invert Sugar Is Opposite in Character to That of α-Methyl Glucoside.—In the first paper of this series, Nelson and Freeman (2) pointed out that, although α-methyl glucoside retards

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the hydrolysis of sucrose by invertase, still, when equation (1) is applied to the results obtained from the hydrolysis of a 10 per cent sucrose solution containing glucoside, the calculated values for N show an increase as the reaction proceeds (Table I C). This increase in N is just the opposite to the decrease in N in the case

TABLE I.

pH 4.67. Temperature 25°C.

A. 10 per cent sucrose.				B. 10 per cent sucrose + 6 gm. of invert sugar.			
Time.	Rotation.	Hydrolysis.	N	Time.	Rotation.	Hydrolysis.	N
min.		*per cent*		*min.*		*per cent*	
0	22.30°			0	18.40°		
15	18.70	12.46	599	7.5	17.04	4.71	447
30	15.20	24.57	601	15	15.67	9.45	444
45	11.95	35.81	602	45	10.58	27.06	435
60	9.00	46.02	601	80	5.50	44.63	425
80	5.51	58.10	601	115	1.42	58.75	416
110	1.40	73.32	603	150	−1.75	69.72	416
∞	−6.60			∞	−10.50		
C. 10 per cent sucrose + 0.125 gm. of glucoside.				D. 10 per cent sucrose + 0.125 gm. of glucoside + 6 gm. of invert sugar,			
0	22.90			0	19.08		
7.5	21.70	4.15		15	17.08	6.92	327
15	20.49	8.34	395	60	11.38	26.66	328
45	15.71	24.88	407	105	6.20	44.57	331
80	10.50	42.91	416	140	2.98	56.07	328
120	5.32	60.83	426	180	−0.43	67.51	330
150	2.13	71.87	438	∞	−9.82		
∞	−6.00						

where invert sugar has been added to the hydrolyzing sucrose solution. In other words, when the glucoside is the retardant, then (if N is taken as a measure of the rate of hydrolysis) its retarding influence becomes less and less as the reaction progresses, while when added invert sugar acts as the retardant, its retarding influence increases as the reaction continues.

Relative Amounts of Glucoside and Invert Sugar Required to Offset Each Other's Influence.—Since the two retardants, glucoside and invert sugar, affect the hydrolysis in different ways, it was thought that very likely if these retardants were present in certain definite amounts with respect to each other, then the course of the reaction might again tend to become normal. The course of a hydrolysis is considered as normal, when equation (1) gives constant values for N, or when the per cent hydrolyzed—time curve (like those in Fig. 1) is similar in shape to a corresponding curve representing the hydrolysis of a 10 per cent sucrose solution containing no added

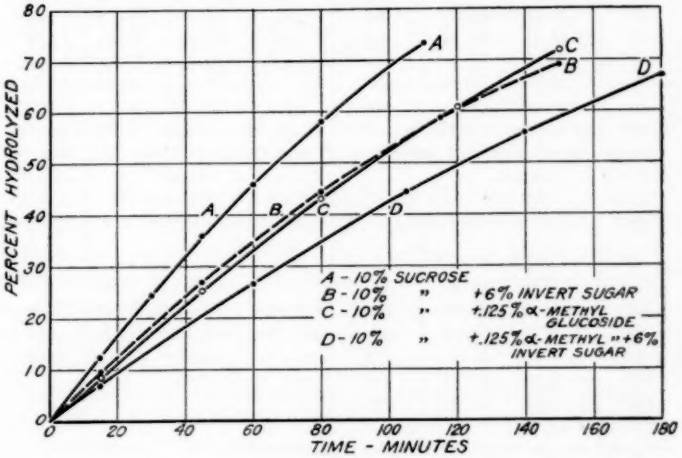
PERCENT HYDROLYZED
TIME - MINUTES
A - 10% SUCROSE
B - 10% " +6% INVERT SUGAR
C - 10% " +.125% α-METHYL GLUCOSIDE
D - 10% " +.125% α-METHYL " +6% INVERT SUGAR

FIG. 1.

retardant. When a hydrolysis curve can be made superimposable upon another such curve by multiplying the time coordinates by some number, then the two curves are considered to be similar in shape.

By a series of trial mixtures of invert sugar and glucoside, it was found that when the two retardants were present in amounts of 6 gm. and 0.125 gm. per 100 cc., respectively, then the course of the hydrolysis tended to become normal as shown by the values for N in Table I D. And it was also found that when only 3 gm. of invert sugar were added per 100 cc. then only half as much, or

TABLE II.

A.
10 per cent sucrose + 0.0625 gm. of glucoside + 3 gm. of invert sugar.
pH 4.64.

Time.	Rotation.	Hydrolysis.	N
min.		*per cent*	
0	24.48°		
10	20.15	12.81	918
20	16.00	25.09	922
30	12.12	36.57	924
40	8.58	47.04	924
55	4.00	60.59	925
70	0.42	71.18	923
∞	−9.32		

B.
10 per cent sucrose + 0.125 gm. of glucoside + 6 gm. of invert sugar.
pH 2.98.

Time.	Rotation.	Hydrolysis.	N
min.		*per cent*	
0	22.38°		
10	19.24	9.29	661
20	16.15	18.43	667
40	10.42	35.38	668
60	5.40	50.24	667
80	1.16	62.78	668
100	−2.24	72.84	671
∞	−11.42		

C.
10 per cent sucrose + 0.125 gm. of glucoside + 6 gm. of invert sugar.
pH 6.73.

0	22.57		
20	17.72	14.35	514
40	13.15	27.87	516
61	8.73	40.95	516
80	5.16	51.51	516
100	1.91	61.12	515
130	−2.05	72.84	517
∞	−11.23		

D.
10 per cent sucrose + 0.125 gm. of glucoside + 6 gm. of invert sugar.
pH 4.64.

0	22.31		
22.5	15.94	18.85	608
46.5	9.77	37.10	606
101.75	−1.13	69.35	609
117.17	−3.08	75.12	605
∞	−11.49		

E.
pH 4.65. 2 per cent sucrose.

0	4.46		
10	3.40	18.34	
20	2.44	34.95	
32	1.46	51.90	
40	0.94	60.90	
55	0.16	74.39	
90	−0.80	91.00	
∞	−1.32		

F.
2 per sucrose + 0.125 gm. of glucoside + 6 gm. of invert sugar.
pH 4.65.

0	1.39		
10	0.45	16.26	
20	−0.37	30.45	
30	−1.08	42.73	
40	−1.70	53.46	
55	−2.43	66.09	
100	−3.69	87.89	
∞	−6.44		

TABLE II—*Concluded.*

G. pH 4.65. 5 per cent sucrose.				H. 5 per cent sucrose + 0.125 gm. of glucoside + 6 gm. of invert sugar. pH 4.65.			
Time.	Rotation.	Hydrolysis.	*N*	Time.	Rotation.	Hydrolysis.	*N*
min.		*per cent*		*min.*		*per cent*	
0	11.13°			0	8.01°		
10	8.64	17.26		20	5.53	17.16	
20	6.33	33.22		40	3.27	32.80	
30	4.29	47.34		60	1.29	46.51	
40	2.56	59.32		80	−0.41	58.27	
55	0.52	73.43		100	−1.79	67.82	
95	−2.20	92.25		180	−4.95	89.70	
∞	−3.32			∞	−6.44		

0.0625 gm., glucoside was required to make the hydrolysis proceed along the curve followed when the retardants are absent (see Table II A). It was also found that this ratio of the two retardants was independent of the hydrogen ion concentration and of the concentration of the invertase causing the hydrolysis. The last two statements are borne out by the experimental data given in Table II B, C, and D.

To see whether the ratio between the amounts of the two retardants present was independent of the sucrose concentration, hydrolyses were run on 2 and 5 per cent sucrose solutions besides the 10 per cent mentioned above, both with and without the presence of retardants (see Table II E, F, G, and H). Since the equation of Nelson and Hitchcock is not applicable to sucrose solutions of these concentrations, a graphical method had to be used instead. This method consisted in plotting the per cent hydrolyzed as ordinates against the corresponding time as abscissæ. When the time coordinates of the curves obtained in this way, representing hydrolyses in the presence of the retardants, were multiplied by a given number, then it was found that these curves become superimposable upon the corresponding curves representing hydrolyses identical in all respects except that the retardants were absent. This shows that the above ratio of glucoside to invert sugar tends

to give a normal hydrolysis curve with sucrose concentrations varying from 2 gm. to 10 gm. per 100 cc. of solution.

Glucoside and Invert Sugar Neutralize Each Other's Influence on Relationship between Sucrose Concentration and Rate of Hydrolysis.—Nelson and Freeman also pointed out that the relation between sucrose concentration and rate of hydrolysis was affected by the presence of α-methyl glucoside. They showed this in the following way. Several solutions containing different amounts of sucrose were hydrolyzed under the same conditions of temperature, hydrogen ion concentration, and amount of invertase. In order to ascertain the relative initial rates of hydrolysis of these various

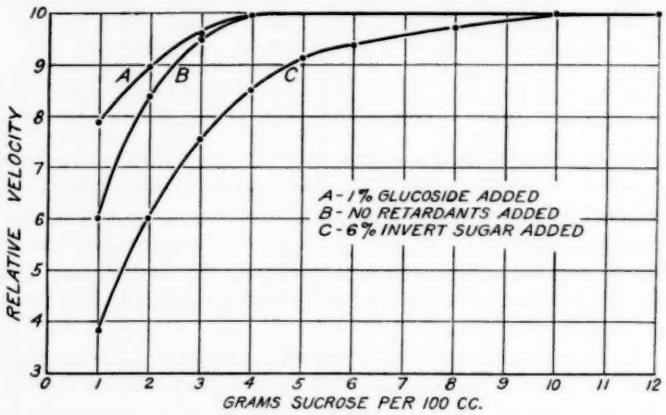

FIG. 2.

sucrose solutions, the results obtained in each individual hydrolysis were plotted as per cent hydrolyzed against time, and tangents drawn to the origin of these hydrolysis curves. The values of these tangents were then taken as the relative initial rates of hydrolysis of their respective solutions. This relationship between the initial velocities and the concentration of sucrose is shown graphically by Curve B in Fig. 2. Curve A in the same figure was obtained in the same way as that used for Curve B, except each of the various sucrose solutions contained a constant amount, 1 per cent, of added glucoside. For the sake of comparison of the initial rates of hydrolysis, the maximum rates in each

set of experiments, *i.e.* the hydrolysis in the absence and presence of glucoside, were put equal to 1000 and the lesser rates as fractions thereof. In other words, the change in velocity with respect to the change in sucrose concentration in the two cases was put on the same relative scale. By comparing the shapes of the two curves, A and B in Fig. 2, it will be seen that the retardation of the hydrolysis due to the presence of a constant amount of α-methyl glucoside increases as the sucrose concentration increases,

TABLE III.

A. pH 4.65. Temperature 25°C.

Sucrose.	Initial velocity.	Relative velocity.
per cent		
1	0.067	604
2	0.093	838
3	0.106	955
4	0.1097	988
4.25	0.110	991
5	0.111	1000
6	0.111	1000
7	0.111	1000
B. Hydrolyses in presence of 6 per cent invert sugar.		
1	0.048	380
2	0.076	605
3	0.094	755
4	0.106	850
5	0.114	915
6	0.117	935
8	0.121	970
10	0.125	1000

or the retardation is least in the more dilute sucrose solutions and reaches a maximum when the sucrose concentration has reached a value corresponding to a maximum rate of hydrolysis.

To see whether invert sugar influences the relation between the rate of hydrolysis and the concentration of sucrose in the opposite way from that of glucoside, a series of hydrolyses was run similar to the series run by Nelson and Freeman for Curve A in Fig. 2, except that 6 per cent of invert sugar was added to each of the different sucrose solutions instead of 1 per cent of glucoside. The

relative initial rates of hydrolysis obtained from this set of hydrolyses, Table III B, have been plotted, giving Curve C in Fig. 2. By comparing the shape of Curve C with those of A and B in the same figure, it becomes evident that the invert sugar affects the relation between the rate of hydrolyses and sucrose concentration in a different manner from the effect due to glucoside. The invert sugar exerts its greatest retardation in the dilute sucrose solutions, which is just the opposite effect to that observed in the case when glucoside is the retardant.

It has been shown above that when the relative amounts of glucoside and invert sugar added to a 10 per cent sucrose solution undergoing hydrolysis bear a certain ratio with respect to one another, then the course of the hydrolysis tends to become normal; *i.e.*, when the results from the hydrolyses are substituted in equation (1), practically constant values for N are obtained. When this same ratio of glucoside and invert sugar was used to study their combined effect on the relationship between the rate of hydrolysis and concentration of sucrose, it was found that here again the two retardants in these proportions offset each other's influence. The procedure followed in these experiments was the same as that used in determining the relative initial velocities when the influence of each retardant on the relation between the velocity and sucrose concentration was studied separately. The results obtained from these experiments, *i.e.* the initial rates of hydrolysis when the mixture of the two retardants was used, are given in Table IV. If these values are plotted similarly to the results represented by curves in Fig. 2, it will be found that the shape of the resulting curve coincides with Curve B in that figure. It will be recalled that Curve B represents the change in the initial rate of hydrolysis for increasing sucrose concentration in the absence of retardants. Since the curve representing the change in initial rate with increasing sucrose concentration is superimposable upon Curve B, this means that when the two retardants are both present in the hydrolyzing sucrose solution they exert a peculiar neutralizing effect upon one another.

Effect of Invert Sugar on the pH Optimum Curve.—Nelson and Freeman examined the influence of α-methyl glucoside on the relation between hydrogen ion concentration and rate of hydrolysis of sucrose by invertase from brewers' yeast. This was done by

TABLE IV.

Hydrolyses in the presence of 6 per cent invert sugar and 0.125 per cent α-methyl glucoside.

Sucrose.	Initial velocity.	Relative velocity.
per cent		
1	0.0187	593
2	0.0263	838
3	0.0296	940
4	0.0318	1000
5	0.0313	1000
6	0.0314	1000
8	0.0316	1000

TABLE V.

A. 6 per cent sucrose. Invertase BW. Temperature 25°C.

pH	Initial velocity.	Relative velocity.
2.90	0 143	877
3.15	0.148	908
3.75	0.156	957
4.21	0.160	982
4.64	0.163	1000
5.27	0.161	988
5.99	0.144	883
6.31	0.134	822
6.70	0.111	681
6.78	0.105	644

B. 6 per cent sucrose and 6 per cent invert sugar. Invertase BW. Temperature 25°C.

2.80	0.100	855
3.06	0.104	889
3.65	0.111	949
4.17	0.114	975
4.64	0.117	1000
5.24	0.114	975
5.95	0.103	880
6.31	0.094	803
6.70	0.083	710
6.79	0.080	684

determining the initial rates of hydrolysis of several 10 per cent sucrose solutions, all containing 1 per cent glucoside, at different hydrogen ion concentrations, and then plotting the values thus obtained against their corresponding hydrogen ion concentrations. This curve was then compared with the curve, the data for which were obtained in the same way except that retardant was ab-

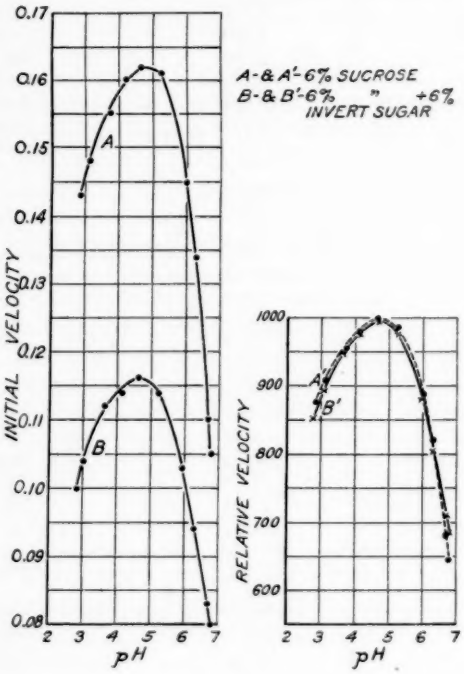

FIG. 3.

sent, and it was found that by multiplying the ordinates of one curve by a suitable constant the two curves then became superimposable.

In the present study the influence of invert sugar on the optimum pH curve has been investigated. The initial rates of hydrolyses, determined as described above, were run on 6 per cent

sucrose solutions at various hydrogen ion concentrations, both with and without 6 per cent added invert sugar. The results obtained are given in Table V A and B, and are shown graphically in Fig. 3. It will be seen that the optimum point is unchanged, *i.e.* it still remains at pH 4.5 to 4.7, and that here again the two curves, in the presence and absence of retardant, may be made superimposable by multiplying the ordinates of one by a suitable constant.

It would appear, then, that invert sugar and α-methyl glucoside have at least one point of similarity, in that their retardation is not affected by the acidity of the solution, nor does either one change the optimum point of the hydrolysis of sucrose by invertase at these concentrations.

SUMMARY.

1. It has been found that, when α-methyl glucoside and invert sugar are present in the ratio of 0.125 to 6 per cent, the hydrolysis of sucrose by yeast invertase tends to follow the same course as when the two retardants are absent, and that the effect of the mixture is the same as that of using less concentration of the enzyme.

2. It has been found that invert sugar and α-methyl glucoside tend to neutralize each other's influence on the relationship between rate of hydrolysis and sucrose concentration.

3. It has been found that retardation due to invert sugar, like that due to α-methyl glucoside is independent of the hydrogen ion concentration.

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STUDIES ON RACEMIZATION.

III. ACTION OF ALKALI ON GLYCYL-LEVOALANYL-GLYCINE AND ON GLYCYL-GLYCYL-LEVOALANYL-GLYCINE.

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(Received for publication, March 24, 1926.)

In two previous communications[1] we have reported on the action of alkali upon three different ketopiperazines, upon two dipeptides, and upon one tripeptide. The bearing of the work on the problem of racemization of proteins on the one hand, and on the theories of the structure of the protein molecule, on the other, have been discussed in those communications. It was recognized, however, that final conclusions concerning the significance of the observations will be reached only after more data have been accumulated on the action of alkalies upon ketopiperazines of various amino acids, upon peptides of greater complexity and upon proteins.

In the previously reported observations the time of the action of alkali did not exceed 72 hours. In that interval the three ketopiperazines which were used in our experiments were all hydrolyzed to the dipeptides. Hence, for the purpose of the investigation of the action of alkali upon ketopiperazines, the intervals were of sufficient duration.

Peptides, on the other hand, depending upon the concentration of the alkali, were either partially hydrolyzed or remained intact. Inasmuch as in the experiments on racemization of proteins the latter were exposed to the action of alkali for several weeks, it seemed desirable to observe the effect of prolonged action of alkali upon polypeptides.

In the present communication the results are reported, first,

[1] Levene, P. A., and Pfaltz, M. H., *J. Biol. Chem.*, 1925, lxiii, 661; *J. Gen. Physiol.*, 1925, viii, 183.

on the action of alkali during a short interval upon a tetrapeptide, glycyl-glycyl-levoalanyl-glycine and, further, on the prolonged action of alkali upon the tripeptide glycyl-levoalanyl-glycine and upon the tetrapeptide glycyl-glycyl-levoalanyl-glycine.

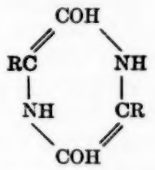
The general procedure in the present experiments was identical with that previously described.

The results of the observations were the following. A tenth molar solution of tetrapeptide containing 1, 2, or 10 equivalents of alkali was kept at 15°C. for 48 hours. In the first two experiments the optical rotation of the solution remained unchanged at the end of 48 hours. On hydrolysis of the tetrapeptide, the resulting alanine was found to possess full activity. In the experiment with 10 equivalents of alkali, the optical rotation dropped to 36 per cent of its original value at the end of 48 hours. This drop was due entirely to a hydrolysis of the peptide, inasmuch as the alanine obtained from it on hydrolysis possessed its full activity.

In the experiments on prolonged action of alkali the following observations were made. After 39 days a tenth molar solution of the tetrapeptide containing 2 equivalents of alkali on hydrolysis yielded alanine possessing its full activity. After 19 days a tenth molar solution of the tetrapeptide containing 5 equivalents of alkali on hydrolysis yielded alanine showing a rotation $[\alpha]_D^{20} = -13.9°$, thus only slightly below the full rotation. After 39 days in the presence of 10 equivalents of alkali, the tetrapeptide yielded alanine with a rotation of $[\alpha]_D^{20} = -13.0°$. Finally, a 0.125 M solution of tripeptide containing 4 equivalents of alkali after 50 days gave, on hydrolysis, alanine with a rotation $[\alpha]_D^{20} = -13.5°$. The peptide was hydrolyzed by the alkali to the extent of 80 per cent. The results then are as follows: (1) In an interval of 48 hours, which is sufficient to racemize ketopiperazines, glycyl-levoalanyl-glycine and glycyl-glycyl-levoalanyl-glycine retained their optical activity in the presence of alkali. (2) On prolonged action of alkali there was noted a slight drop in the rotatory power of the active component only when the concentration of the alkali was from four to ten times as great as that of the peptide. It must be added, however, that the extent of racemization never exceeded 10 per cent, as a rule being less, and that in all these cases hydrolysis of the pep-

tides was observed reaching 80 per cent of the original concentration. Thus it is not possible to tell whether the racemization took place before or after hydrolysis.

In our first publication on this subject we have suggested that the racemization of ketopiperazines may be due to the intermediary step of enolization to the following form.



Recently Abderhalden and Schwab[2] made a very important discovery of a new isomeric form of glycyl-glycine anhydride. They ascribe to the new form the above enolic structure. They also point out the possible connection which these enolic forms may have with racemization of proteins.

The synthesis of optically active peptides is a time-consuming undertaking and the progress of the present work with the facilities at our disposal will be very slow. Hence, individual communications will naturally have a fragmentary character.

EXPERIMENTAL.

In view of the fact that the tetrapeptide glycyl-glycyl-levoalanyl-glycine had not been prepared, the details of its preparation and its physical constants and those of the chloracetyl-glycyl-levoalanyl-glycine are here described. The starting point for the tetrapeptide was the tripeptide glycyl-levoalanyl-glycine.

Preparation of Chloracetyl-Glycyl-Levoalanyl-Glycine.—4 gm. of glycyl-levoalanyl-glycine were dissolved in 20 cc. of N sodium hydroxide (1 equivalent) and treated alternately with 2.7 gm. of chloracetyl chloride (1.2 equivalents) and 40 cc. of N sodium hydroxide under cooling, each reagent being added in five equal portions. The solution was then neutralized with 7.2 cc. of 5 N hydrochloric acid and evaporated to dryness under diminished pressure. The residue was extracted with about 100 cc. of hot

[2] Abderhalden, A., and Schwab, E., *Z. physiol. Chem.*, 1926, clii, 88.

absolute alcohol. On cooling the alcohol extract in a freezing mixture, a flocculent precipitate was obtained which consisted of chloracetyl-glycyl-levoalanyl-glycine slightly contaminated with inorganic material. The yield was 4.5 gm. A second crop of 1.0 gm. was obtained by evaporating the alcohol filtrate to dryness, extracting the residue with about 25 cc. of hot absolute alcohol, and cooling as before. The substance contained traces of inorganic material which could not be removed by further recrystallization from absolute alcohol. It is very soluble in water and in hot absolute alcohol, and insoluble in ethyl acetate, chloroform, acetone, ether, benzene, and petrolic ether. It melts at 130°C. with decomposition. For analysis it was dried *in vacuo* over sulfuric acid at 80°C.

0.1000 gm. substance required (Kjeldahl) 10.45 cc. 0.1 N HCl.
0.1350 " " gave (Carius) 0.0698 gm. AgCl.
$C_9H_{14}O_5N_3Cl$. Calculated. N 15.02, Cl 12.88.
Found. " 14.63, " 12.79.

It had an optical rotation of:

$$[\alpha]_D^{19} = \frac{+1.45° \times 100}{1 \times 3} = +48.3°.$$

Preparation of Glycyl-Glycyl-Levoalanyl-Glycine.—10 gm. of chloracetyl-glycyl-levoalanyl-glycine were allowed to stand 2 days at room temperature with 50 cc. of ammonium hydroxide (sp. gr. 0.90). The solution was then concentrated under diminished pressure to a thick syrup. The syrup was heated on the water bath with about 100 cc. of absolute alcohol until crystallization took place. The crystallized material was purified by dissolving it in a minimum amount of hot water and adding two parts of hot absolute alcohol. The substance was still found to contain a trace of inorganic material which could not be completely removed by further recrystallization. It melted at 205° with decomposition.

0.0987 gm. substance: 0.1504 gm. CO_2 and 0.0532 gm. H_2O.
0.0928 " " : (Dumas) 17.1 cc. N at 762.2 mm. and 20°C.
$C_9H_{16}O_5N_4$. Calculated. C 41.51, H 6.20, N 21.54.
Found. " 41.54, " 6.12, " 21.52.

It had an optical rotation of:

$$[\alpha]_D^{25} = \frac{+1.61^\circ \times 100}{1 \times 3} = +53.7^\circ.$$

Action of Alkali of Different Concentrations during a Period of 48 Hours.

1. 0.5700 gm. of glycyl-glycyl-levoalanyl-glycine was dissolved in 20 cc. of 0.1 N sodium hydroxide, the original rotation of the solution being +2.64° (l = 200 mm.). After 48 hours the rotation was +2.57°, thus showing that no hydrolysis took place. For neutralization the solution was taken up in an equal volume of 0.1 N HCl solution. The rotation of the neutral solution was +1.52° or reduced to the original volume the rotation was +3.04°. Hence, the $[\alpha]_D^{20} = +53.3^\circ$, again shows that the peptide was in no way affected by the action of the alkali. Part of the solution was concentrated under reduced pressure to small volume and made up to 10.0 cc. of a solution containing 10 per cent of hydrochloric acid. This solution was hydrolyzed in a sealed tube for 12 hours at 100°C. In this solution the concentration of alanine was 0.706 per cent. Hence the rotation of the resulting alanine was:

$$[\alpha]_D^{20} = \frac{-0.11^\circ \times 100}{1 \times 0.706} = -15.5^\circ.$$

2. In this experiment 0.5700 gm. of the substance was dissolved in 20.0 cc. of 0.2 N sodium hydroxide. The original rotation was +2.60°, after 48 hours +2.56°, and on neutralization the rotation calculated to the original volume was +3.02°. Thus again no hydrolysis was observed. The hydrolyzed solution contained in 10 cc. of the solution 0.0532 gm. of nitrogen. Hence, the concentration of alanine was 0.845 per cent. The optical rotation of the alanine was:

$$[\alpha]_D^{20} = \frac{-0.13^\circ \times 100}{1 \times 0.845} = -15.3^\circ.$$

3. 0.5700 gm. of the same peptide was dissolved in 20 cc. of 1.0 N sodium hydroxide. The original rotation of the solution was +2.30° (l = 200 mm.). After 48 hours the rotation was

+1.48°. On neutralization, and after calculating to the original concentration, the rotation was +1.76°. Thus a considerable hydrolysis took place inasmuch as the resulting alanine had its full rotation. The hydrolyzed solution contained 0.0569 gm. of nitrogen in 10 cc. Hence the concentration of alanine was 0.8995 per cent and its optical rotation was:

$$[\alpha]_D^{20} = \frac{-0.14^\circ \times 100}{1 \times 0.8995} = -15.5^\circ.$$

4. In view of the fact that the final absolute readings were rather small and in view of the fact that the intermediate readings of the rotation consumed an appreciable part of the solution, the following experiment was performed. 0.5700 gm. of the substance was dissolved in 20 cc. of 0.2 N sodium hydroxide and allowed to stand 48 hours. At the end of that time the solution was neutralized and hydrolyzed. After hydrolysis, 10 cc. of the solution contained 0.122 gm. of nitrogen; hence the concentration of alanine was 1.96 per cent. The specific rotation was:

$$[\alpha]_D^{20} = \frac{-0.60^\circ \times 100}{2 \times 1.96} = -15.3^\circ.$$

Prolonged Action of Alkali.

1. 0.5700 gm. of the tetrapeptide was dissolved in 20 cc. of 0.5 N sodium hydroxide. That the tetrapeptide was undergoing gradual hydrolysis is seen from the changes in the optical rotation of the solution. These changes could not be interpreted by racemization of the peptide inasmuch as the alanine obtained after hydrolysis had a specific rotation only slightly lower than the normal.

The changes of rotation of the solution were as follows:

Time.	$[\alpha]_D^{20}$
days	
0	+2.48°
2	+2.09°
9	+1.35°
13	+1.25°
16	+1.05°
18	+1.05°

The solution was then hydrolyzed in the usual way. The final solution contained 0.0720 gm. of nitrogen in 10 cc. Hence the concentration of alanine was 1.15 per cent, and the specific rotation was:

$$[\alpha]_D^{20} = \frac{-0.32^\circ \times 100}{2 \times 1.15} = -13.9^\circ.$$

2. 0.7100 gm. of the tetrapeptide was dissolved in 25 cc. of 0.2 N sodium hydroxide and allowed to stand 39 days. At the end of that time the solution was neutralized, concentrated, and hydrolyzed in the usual way. The final solution contained 0.1281 gm. of nitrogen in 10 cc. Hence the concentration of alanine was 2.08 per cent. The specific rotation of the alanine was:

$$[\alpha]_D^{20} = \frac{-0.62^\circ \times 100}{2 \times 2.08} = -14.9^\circ.$$

3. 0.7100 gm. of the tetrapeptide was dissolved in 25 cc. of 0.5 N sodium hydroxide. After 39 days the solution was neutralized and then hydrolyzed in the usual way. The final solution contained 0.1327 gm. of nitrogen in 10 cc. Hence the concentration of alanine was 2.11 per cent. The specific rotation of the alanine therefore was:

$$[\alpha]_D^{20} = \frac{-0.55^\circ \times 100}{2 \times 2.11} = -13.0^\circ.$$

4. 0.5000 gm. of the tripeptide glycyl-levoalanyl-glycine was dissolved in 20 cc. of 0.5 N sodium hydroxide. After 50 days the solution was neutralized. Part of it was concentrated and hydrolyzed in the usual way. The solution contained 0.032 gm. in 10 cc. Hence the concentration of alanine was 0.674 per cent. The specific rotation of the alanine was:

$$[\alpha]_D^{20} = \frac{-0.18^\circ \times 100}{2 \times 0.67} = -13.3^\circ.$$

PLANT PHOSPHATIDES.

II. LECITHIN, CEPHALIN, AND SO CALLED CUORIN OF THE SOY BEAN.

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The present work is a continuation of the previous publication[1] on the commercial product obtained from soy bean and furnished us through the courtesy of Dr. H. Bollmann of the Hanseatische Mühlenwerke. The process of preparation of the commercial product has been continually modified by the Mühlenwerke and finally we were furnished with three fractions differing from one another in their solubility in alcohol.

From the fraction possessing the greatest solubility in alcohol it was possible to obtain lecithin with the general properties of the soy bean lecithin previously described by us. It has been employed for extending the data on the components of this lecithin fraction.

In the previous work the following facts were established. (1) That the lecithin fraction contained two saturated fatty acids; namely, stearic and palmitic. (2) That it contained three unsaturated fatty acids. Of these, two were identified as linolic and linolenic. There were found indications that the third acid was oleic. The acid, however, was not identified then. Thus, the data on the unsaturated fatty acids were incomplete.

Besides, the character of the glycerophosphoric acid entering into the structure of the plant lecithin has not been investigated.

The present investigation represents an effort to obtain the lacking information. The oleic acid was isolated in the form of the free acid, which melted at 12°C. and had an iodine number

[1] Levene, P. A., and Rolf, I. P., *J. Biol. Chem.*, 1924–25, lxii, 759.

of 88. In addition, the acid was identified by conversion into elaidic acid melting at 44.5°C.

The problem of the character of the glycerophosphoric acid[2] of plant lecithin presented considerable experimental difficulty, particularly when, not the purified lecithin, but the crude product, was used as starting material. The difficulty arose from the fact that samples of the barium salts analytically resembling the composition of glycerophosphoric acid possessed a dextrorotation instead of the levorotation which is characteristic for the animal glycerophosphoric acid. The assumption of a dextrorotatory glycerophosphoric acid for the plant lecithin seemed inconsistent with the fact that hydrogenated plant lecithin rotated in the same direction as hydrogenated lecithin of animal origin. It was subsequently found that the plant lecithin even after purification through the cadmium salt still contained an impurity of a carbohydrate nature. This impurity was particularly abundant in the crude lecithin. The carbohydrate possesses a high dextrorotation, so that even small admixtures of it hid the levorotation of the glycerophosphoric acid. Only after a great many purifications was it possible to bring out the levorotation of the glycerophosphoric acid.

The nature of the carbohydrate has not been investigated. One characteristic of it, however, is worthy of note. When tested with naphthoresorcinol in the usual manner, it gave a purple color resembling that which pentoses give with orcinol. The absorption bands of the pigment in amyl alcoholic solution were the same as those of the pentose orcinol pigment. The carbohydrate gave a negative color test with orcinol.

Another observation which we wish to record in this communication is the presence in the purified phosphatide furnished to us by Dr. Bollmann of a material resembling in its elementary composition the so called cuorin of Erlandsen.

According to information furnished us by Dr. Bollmann, only neutral organic solvents are employed in the preparation of the commercial phosphatides.

In the case of the plant phosphatides, also, the cuorin is a product of partial disintegration of lecithin and cephalin. It can be fractionated into a portion free from non-amino nitrogen.

[2] Levene, P. A., and Rolf, I. P., *J. Biol. Chem.*, 1919, xl, 1.

The product is a light yellow solid which does not change in appearance on long standing. Its components are identical with those of cephalin, when free from non-amino nitrogen. It differs from cephalin by a smaller proportion of the unsaturated fatty acids and by a smaller proportion of the base aminoethanol.

This substance differs from the animal cuorin by the greater simplicity of the admixture. Levene and Komatsu[3] have shown that the animal cuorin contained a considerable proportion of lysolecithin and lysocephalin in addition to fragments lacking in both base and unsaturated fatty acids. In the plant cuorin only the latter products are found.

It is not known whether the material is a product of enzyme action within the bean or an artificial product arising in the process of manufacture of the commercial lecithins.

EXPERIMENTAL.

A. Lecithin Fraction.

I. Hydrolecithin.

5 gm. of the lecithin described in a previous publication[4] were hydrogenated with colloidal palladium. The hydro derivative had the following rotation in chloroform solution.

$$[\alpha]_D^{20} = \frac{+0.62^\circ \times 100}{1 \times 9} = +6.9^\circ.$$

II. Products of Hydrolysis of Lecithin.

1. Glycerophosphoric Acid.—An ethereal solution of lecithin was emulsified in water. To the emulsion one and a half parts by weight of barium hydroxide were added and the mixture was allowed to stand on the water bath for 2 hours. Through the filtrate from the barium salts carbon dioxide gas was passed and the filtrate from barium carbonate was concentrated to a small volume, neutralized with acetic acid, and treated with a solution of neutral lead acetate. The precipitate was the lead salt of crude glycerophosphoric acid. From the filtrate, on addition of basic lead acetate and of barium hydroxide, a precipitate settled

[3] Levene, P. A., and Komatsu, S., *J. Biol. Chem.*, 1919, xxxix, 83.
[4] Levene, P. A., and Rolf, I. P., *J. Biol. Chem.*, 1925, lxv, 545.

out which contained the carbohydrate. The precipitated barium salt was purified by conversion into the lead salt; the lead salt of the glycerophosphoric acid was decomposed and reconverted into the lead salt. The operation was repeated as many times as necessary to obtain a levorotatory solution. From this solution the barium salt was precipitated by means of alcohol. As many as fifteen reprecipitations with lead acetate often were required in order to prepare a levorotatory sample. For analysis the substance was dried at reduced pressure at the temperature of toluene vapor.

0.1018 gm. substance: 0.0472 gm. CO_2 and 0.0232 gm. H_2O.
0.0929 " " : 0.0672 " $BaSO_4$.
0.1858 " " : 0.0644 " $Mg_2P_2O_7$.
$C_3H_7O_6P.Ba$. Calculated. C 11.71, H 2.29, P 10.10, Ba 44.68.
Found. " 12.64, " 2.55, " 9.66, " 42.56.

The specific rotation of the substance was:

$$[\alpha]_D^{20} = \frac{-0.10^\circ \times 100}{4 \times 4} = -0.63^\circ.$$

2. Oleic Acid.—The unsaturated acid fraction obtained from the acid hydrolysis of soy bean lecithin was converted into a lead salt, and all material insoluble in ether removed. The acids were liberated by means of hydrogen sulfide and dissolved in methyl alcohol. From this solution they were precipitated as the barium salts, and these were fractionated with a mixture of benzene containing 5 per cent of 95 per cent ethyl alcohol. The insoluble barium oleate was converted to the free acid which melted at 12°. 0.2050 gm. of substance absorbed 15.0 gm. of I_2.

$C_{18}H_{34}O_2$. Calculated. Iodine No. = 90.
Found. " " = 88.

This acid, in the presence of nitrous acid, was converted into an acid which, after recrystallization from alcohol, melted at 44°. The melting point of elaidic acid is 44.5°.

B. Fraction Resembling Cuorin.

I. Isolation and Composition.

After the removal of all the alcohol-soluble material, the acetone-insoluble portion of the crude soy bean lipoids, on treat-

ment with glacial acetic acid, could be fractionated into two parts. The one, insoluble in glacial acetic acid, corresponded closely with the lipoid fraction designated by previous workers as cuorin, while the more soluble material was the cephalin fraction, analyzed in a previous report. After the removal of the cephalin had been completed by repeated extraction with warm acetic acid, the insoluble fraction was hardened with acetone. At this stage it was a gray-white, amorphous powder resembling the cerebrosides. It showed no tendency to become sticky or to discolor on exposure to the air, provided that the unsaturated cephalin material had been thoroughly extracted. It was insoluble in alcohol, acetone, methyl ethyl ketone, and glacial acetic acid but was very soluble in chloroform. It absorbed bromine and iodine, gave a positive orcinol test, and gave the following analysis.

No. 117. 0.1038 gm. substance: 0.2216 gm. CO_2, 0.0828 gm. H_2O, and 0.0120 " ash.
0.1932 " " required (Kjeldahl) 1.15 cc. 0.1 N acid.
0.2899 " " : (fusion) 0.0362 gm. $Mg_2P_2O_7$.
No. 118. 0.1943 " " required (Kjeldahl) 1.15 cc. 0.1 N acid.
0.2915 " " : (fusion) 0.0372 gm. $Mg_2P_2O_7$.
No. 119. 0.1880 " " required (Kjeldahl) 1.25 cc. 0.1 N acid.
0.2820 " " : (fusion) 0.0566 gm. $Mg_2P_2O_7$.
No. 120. 0.1886 " " required (Kjeldahl) 1.35 cc. 0.1 N acid.
0.2829 " " : (fusion) 0.0448 gm. $Mg_2P_2O_7$.
Found. No. 117. C 58.21, H 8.92, N 0.83, P 3.47.
" 118. " 0.82, " 3.55.
" 119. " 0.93, " 5.59.
" 120. " 1.00, " 4.40.

The material was practically free from lecithin or from fragments of it as may be seen from the following analysis.

No. 111. 0.1034 gm. substance: 0.2226 gm. CO_2, 0.0834 gm. H_2O, and 0.0124 " ash (11.99 per cent).
0.1926 " " required (Kjeldahl) 1.10 cc. 0.1 N acid.
0.2889 " " : (fusion) 0.0368 gm. $Mg_2P_2O_7$.
2 gm. were hydrolyzed with 10 per cent HCl.
5 cc. required: (Kjeldahl) 1.06 cc. 0.1 N acid.
2 " " : (Van Slyke) 1.02 cc. N_2 at 764 mm., 20°.
0.2747 gm. substance absorbed 0.1566 gm. I_2.
Found. No. 111. C 58.70, H 9.02, N 0.79, P. 3.55.

$$\frac{\text{Amino N}}{\text{Total N}} = \frac{95}{100}.$$ Iodine No. = 58.

In chloroform solution its rotation was:

$$[\alpha]_D^{20} = \frac{+1.60° \times 100}{1 \times 10} = +16°.$$

After standing for a month its iodine value had fallen to 28.

Attempts to purify this substance by solution in $CHCl_3$ followed by precipitation with alcohol, acetone, and glacial acetic acid gave rise to materials having the compositions given below, which are seen to approximate closely the appended tables quoted by Maclean[5] for the analytical data on cuorin.

Observer.	Source of material.	C	H	N	P	N:P ratio.
Erlandsen.	Ox heart.	61.46	8.92	1.02	4.43	1:2
	" "	61.52	9.09	1.03	4.52	1:2
Maclean.	Horse kidney.	60.4	10.2	1.04	4.45	1:2
	Egg yolk.	59.12	9.44	0.812	3.59	1:2
Baskoff.	Liver.	61.12	8.95	1.23	4.00	1:1.5

II. Products of Hydrolysis.

1. Saturated Fatty Acids.—40 gm. of No. 111 were hydrolyzed for 8 hours with 10 per cent HCl. The cake of fatty acids was dissolved in ether, washed thoroughly with water, dried, and concentrated. The crude, mixed acids, weighing 25 gm., had an iodine value of 99, and a molecular weight of 313. They were dissolved in a minimal quantity of methyl alcohol, from which the saturated acids crystallized. By the usual methyl ester fractionation two acids were isolated having the following analyses, melting points, and molecular weights.

No. 129. 0.1000 gm. substance: 0.2762 gm. CO_2 and 0.1124 gm. H_2O.
0.4859 " " required 18.8 cc. 0.1 N NaOH.
No. 130. 0.0878 gm. substance: 0.2444 gm. CO_2 and 0.1022 gm. H_2O.
0.8032 " " required 28.6 cc. 0.1 N NaOH.
Calculated. $C_{16}H_{32}O_2$. C 74.92, H 12.58, mol. wt. = 256, m.p. 63–64°.
$C_{18}H_{36}O_2$. " 75.93, " 12.78, " " = 284, " 70–71°.
Found. No. 129. " 75.31, " 12.58, " " = 259, " 63°.
" 130. " 75.90, " 13.00, " " = 281, " 70–71°.

[5] Maclean, H., Lecithin and allied substances, Monograph on biochemistry, London, 1918.

2. Unsaturated Fatty Acids.—The fatty acids which had remained in solution in the methyl alcohol were precipitated by the addition of a methyl alcoholic solution of lead acetate. The salts of the unsaturated acids were extracted by means of ether, and were converted by means of H_2S into the free acids. The liquid acids obtained from this solution had a molecular weight of 311 and an iodine number of 156. The acids were brominated in the usual way, and the resulting bromo acids were fractionated by the use of ether.

Two acids were separated having the following properties.

No. 131.	0.0532 gm. substance:	(Carius) 0.0790 gm. AgBr.
No. 132.	0.1056 " "	: 0.1408 gm. CO_2 and 0.0538 gm. H_2O.
	0.1048 " "	: (Carius) 0.1354 gm. AgBr.
Calculated.	$C_{18}H_{36}O_2Br_6$.	C 28.49, H 3.99, Br 63.26, m.p. = 180–181°.
	$C_{18}H_{32}O_2Br_4$.	" 36.01, " 5.38, " 53.28, " = 113–114°.
Found.	No. 131.	" 63.20, " = 180–181°.
	" 132.	" 36.36, " 5.70 " 54.98, " = 114°.

The attempt to isolate oleic acid has thus far been unsuccessful.

3. Glycerophosphoric Acid.—For the preparation of glycerophosphoric acid the same procedure had to be employed as in the case of lecithin. The proportion of the carbohydrate impurity was much greater than in the case of lecithin. The barium salt was dried under diminished pressure at the temperature of toluene vapor and analyzed as follows:

0.1022 gm. substance:	0.0512 gm.	CO_2 and 0.0244 gm. H_2O.
0.0941 " "	: 0.0708 "	$Mg_2P_2O_7$.
0.1882 " "	: 0.0554 "	$BaSO_4$.
$C_3H_7O_6P.Ba$.	Calculated.	C 11.71, H 2.29, P 10.10, Ba 44.68.
	Found.	" 13.66, " 2.67, " 8.69, " 44.27.

Its optical rotation was as follows:

$$[\alpha]_D^{20} = \frac{-0.10° \times 100}{2 \times 5} = -1.00°.$$

4. Base.—From the mother liquors of the acid hydrolysis of No. 111 the base was isolated in accordance with the directions given by Levene and Ingvaldsen.[6] Since 94 per cent of the

[6] Levene, P. A., and Ingvaldsen, T., *J. Biol. Chem.*, 1920, xliii, 356.

nitrogen was present as amino nitrogen, no attempt was made to isolate choline. The addition of gold chloride to the concentrated acidified solution was followed, after the usual desiccation, by the separation of the typical large orange crystals of aminoethanol gold chloride. These, after recrystallization from dilute hydrochloric acid, softened at 185° and melted at 190°.

0.0947 gm. substance: (ignition) 0.0468 gm. gold.
$C_2H_7ON.HAuCl_4$. Calculated. Au 49.17.
Found. " 49.41.

C. Cephalin Fraction.

An emulsion of the crude material was taken up in hot 70 per cent alcohol containing a small amount of benzene. The residue was dissolved in hot alcohol. From the solution on standing a precipitate settled out. This material was dissolved in an equal part (by weight) of glacial acetic acid. The thick liquid was poured into a large excess of glacial acetic acid. A precipitate formed. The supernatant liquid was decanted, concentrated to small volume, and poured into 98 per cent alcohol. A precipitate formed (200 gm.) which was extracted with 600 cc. of hot glacial acetic acid. From the extracts a precipitate formed on addition of acetone. This precipitate contained 75.20 per cent of its nitrogen in the form of amino nitrogen. It analyzed as follows:

No. 42. 0.1056 gm. substance: 0.2280 gm. CO_2, 0.0828 gm. H_2O and 0.0128 " ash.
0.2898 " " : (fusion) 0.0380 gm. $Mg_2P_2O_7$.
0.1932 " " required (Kjeldahl) 1.30 cc. 0.1 N HCl.
Oleyl stearyl cephalin. $C_{41}H_{80}O_8NP$.
Calculated. C 65.80, H 10.77, P 4.15, N 1.87.
Found. " 63.55, " 9.46, " 3.66, " 0.96.

200 gm. of this material were dissolved in ether and a small fraction separated by precipitation with an equal volume of glacial acetic acid. The solution precipitated with acetone yielded 165 gm. of a substance whose ratio of $\frac{\text{amino N}}{\text{total N}} = 78$. Its analysis closely resembled that of No. 42.

This material, again dissolved in 400 cc. of ether, when added

to a mixture of 200 cc. of glacial acetic acid and 200 cc. of 95 per cent alcohol, formed an oil which, when hardened on contact with acetone, consisted of 82 gm. of a material analyzing as follows:

No. 58. 0.1056 gm. substance: 0.2274 gm. CO_2, 0.0820 gm. H_2O and 0.0132 " ash.
0.2926 " " : 0.0396 " $Mg_2P_2O_7$.
0.1950 " " required (Kjeldahl) 1.15 cc. 0.1 N HCl.
Found. C 62.83, H 9.29, P 3.77, N 0.85.

$$\frac{\text{Amino N}}{\text{Total N}} = 84.6$$

From the filtrate, on concentration, an additional 35 gm. of substance settled out as a similar oil of substantially the same analysis.

MAGNESIUM METABOLISM ON PURIFIED DIETS.

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INTRODUCTION.

The present study of magnesium metabolism was undertaken for the purpose of attempting to determine something of the normal ratios of magnesium to calcium and phosphorus in the albino rat, and the extent to which these ratios may be varied by varying their percentages in the diet.

The effects of the injection or ingestion of the salts of these metals upon their excretion from the body has been studied by a number of investigators.

Malcolm (1904–05) found that ingestion of soluble magnesium salts by adult animals caused a loss of calcium, while soluble calcium salts did not produce a similar effect on the excretion of magnesium. Mendel and Benedict (1909–10) studied the excretion of magnesium and calcium after injection of various salts of these metals. They found that following injection of $CaCl_2$ there was an increased excretion of magnesium and an increased excretion of magnesium after injection of $MgCl_2$. Hart and Steenbock (1913) added magnesium to pig's ration and found an increased calcium elimination in the urine. Schiff (1920) found that after subcutaneous administration of magnesium sulfate, there was a loss of calcium with retention of one-half to two-thirds of the magnesium administered. Conti (1921) found that after administration of magnesium sulfate, urinary calcium and magnesium increased. Bogert and McKittrick (1922) found that addition of 6 gm. of magnesium citrate to a diet just maintaining calcium balance in women increased the excretion of calcium and magnesium both in the urine and in the feces. Addition of 6 gm. of calcium lactate per day to the diet led to decided increases in both urinary and fecal calcium. The effect on magnesium excretion was indecisive, but apparently it was also slightly increased. Buckner and Peter (1922) prepared tables of the phosphorus, calcium, and magnesium content of rats from 2 to 40 weeks of age. The percentages of phosphorus and calcium increased with age during

the entire period at about the same rate, and were on an average slightly greater in females than in males. On the other hand, magnesium remained at about the same percentage of body weight. It was also slightly higher in females than in males. Atchley, Loeb, and Benedict (1923) fed 20 to 35 gm. of calcium chloride each day and observed that the calcium excretion was only moderately increased. Whelan (1925) injected hypertonic solutions of magnesium and calcium chlorides and determined the magnesium and calcium content of serum and urine during the subsequent 3 to 6 hour periods. She found there was an immediate rise of the injected element in the serum, followed by a gradual decrease with return to normal in approximately 4 hours. Injection of magnesium chloride caused increased excretion of calcium in the urine, while similar injection of calcium sometimes did and sometimes did not cause increase in excretion of magnesium. Sherman and MacLeod (1925) have recently made a more thorough study of the calcium content of the bodies of rats at different ages, determining variations with sex and diets inadequate as to amounts of protein, calcium, or vitamins A or B.

Experimental Procedure and Technique.

The rats used in these experiments were raised from a single inbred litter, fed two parts of whole wheat and one part of whole milk powder. In each experiment a number of the rats from one litter were placed on the experimental diet and others were used as controls. Part of the latter were killed at the beginning of the experiment and part were fed on control diets during the same period as were the rats on experiment. Each rat was kept in a separate cage with wire mesh bottom. The cages were set over 6 inch fused silica dishes into which the excreta fell and in which they were subsequently ashed. At the close of the experiment the rats were x-rayed in order that changes in bone structure might be observed. The rats were then ashed in silica dishes and the ash analyzed for calcium, magnesium, and phosphorus. From the composition of the rat at the close of the experiment and that of the food and feces, it was possible to estimate the composition of the rat at the beginning of the experiment. This was done in the case of each rat, and in all instances the figures obtained agreed closely with those of normal rats of the corresponding age and sex.

Chemical Methods.

The ash of the entire rat was dissolved in dilute nitric acid and the volume made up to 200 cc. The calcium was determined as follows: To an aliquot part of the solution were added 20 cc. of

2.5 per cent oxalic acid and neutralized with 20 per cent sodium bicarbonate, using brom-phenol blue as indicator. Three drops of glacial acetic acid were added for slight buffer effect and the solution boiled and shaken in a mechanical shaker for half an hour. The precipitate was allowed to stand overnight at room temperature. The solution was filtered and the precipitate washed six times in distilled water, dissolved in 5 per cent sulfuric acid, and titrated against standard potassium permanganate (0.05 N).

For the phosphorus determination a second aliquot part of the original solution was taken and nitric acid and ammonia added alternately until the solution was just acid to brom-phenol blue. It was then heated to 40° and a molybdate solution of ammonium molybdate buffered with ammonium nitrate added. The solution was shaken in the mechanical shaker for 15 minutes and allowed to stand at 40° for an hour. It was then filtered, the precipitate washed ten times with 5 per cent potassium nitrate, dissolved in an excess of 0.1 N sodium hydroxide, and the excess titrated back with 0.1 N hydrochloric acid, using phenolphthalein as indicator. In later determinations the method was slightly modified.

For the magnesium determination, the final filtrate from the calcium determination was heated to boiling and N sodium ammonium phosphate added, followed by 6 N ammonia sufficient in amount to produce approximately a concentration of 1.5 N ammonia in the entire solution. The solution was allowed to stand 2 or 3 hours, the supernatant liquid decanted, the precipitate washed three times with 1.5 N ammonia, filtered on No. 44 Whatman filter paper, heated in an electric furnace until white, cooled in a desiccator, and weighed.

For some of the later experiments, Briggs' (1922) method for the determination of magnesium in blood was employed after it was tested for accuracy.

Diets.

The rats used in the various experiments were fed a stock mixture consisting of

Purified casein	22 parts.
" starch	54 "
Sucrose	20 "

to which were added mixtures of salts and vitamins.

The starch was washed first in dilute HCl, being constantly stirred for several hours with a mechanical stirrer, and afterwards allowed to settle. The HCl was then siphoned off, and the starch washed in the same way about twenty times with distilled water. On analysis this starch contained 4 mg. of calcium, 60 mg. of phosphorus, and 0.44 mg. of magnesium per 100 gm.

Grain curd casein was used which had been precipitated at the isoelectric point from fat-free skim milk, washed many times in water at the isoelectric point, and dried. It was found upon analysis to contain 65 mg. of calcium, 315 mg. of phosphorus, and 16 mg. of magnesium per 100 gm.

Dried spinach was used as the source of vitamin A. The spinach was covered with 95 per cent alcohol in a wide mouthed glass-stoppered bottle and allowed to stand for 2 days. The alcohol was drawn off and the process repeated. In using the extract, the calculation was based on the weight of the original dried spinach. After the vitamin extract was added to the diets, the latter were dried on trays before electric fans. The salts were then added and the mixture placed in containers and shaken in a mechanical shaker for about 2 days to insure thorough mixing. Since the Harris yeast vitamin is difficult to mix, it was added separately to the food for each rat. The food for each rat was weighed into a separate bottle, and after the close of the experiment the portion not eaten was again weighed to determine the exact amount consumed. Any food dropped by the rat fell into the silica dish and was analyzed with the excreta. Some of the wide differences in the ratios of calcium, phosphorus, and magnesium in the excreta are undoubtedly due to admixture of food. No antirachitic vitamin was given.

In Table I is given the composition of eight normal rats (Rats 1 to 8) killed at the beginning of the experiment. They were from the same litters as those subsequently used in some of the experiments and had been raised on the same diet.

Other rats from the same litters were kept during the period of the experiment on Diet I (Rats 9 to 13) and Diet II (Rats 14 to 18). These diets were intended to be normal except for lack of the antirachitic vitamin. These rats served as controls in the experiments recorded below. The amounts of calcium, phosphorus, and magnesium in the salt mixture in Diet II were selected

TABLE I.

Composition of Normal Rat.

Diet No.	Rat No.	Sex.	Age at beginning of experiment.	Age at close of experiment.	Days on experiment.	Weight at beginning of experiment.	Weight at close of experiment.	Gain in weight.	Ash of rat.								
									Ca	P	Mg	Ratios.			Per cent of weight of rat.		
												P:Ca	Ca:Mg	P:Mg	Ca	P	Mg
			days	*days*		*gm.*	*gm.*	*gm.*	*mg.*	*mg.*	*mg.*						
	1	♂	29			48			339	249	20	0.73	16.9	12.4	0.70	0.52	0.042
	2	♀	29			36			271	194	14	0.72	19.3	13.9	0.75	0.54	0.040
	3	♂	31			57			396	309	24	0.78	16.5	12.9	0.70	0.54	0.042
	4	♀	31			42			308	261	21	0.85	14.7	12.5	0.73	0.62	0.050
	5	♂	37			97			695	558	36	0 82	19.3	15.8	0.72	0.58	0.037
	6	♀	37			82			657	510	36	0.77	18.5	14.2	0.80	0.62	0.037
	7	♂	48			112			829	605	46	0.72	17.3	13.2	0.74	0.54	0.040
	8	♀	48			101			838	566	40	0.68	20.9	14.2	0.83	0.56	0.040
I	9–11	♂	37	57	20	77	135	58	998	785	53	0.79	19.0	14.9	0.73	0.58	0.039
	12–13	♀	37	57	20	75	122	47	1037	743	49	0.71	21.1	15.2	0.85	0.61	0.040
II	14–16	♂	37	57	20	67	111	43	855	593	43	0.70	19.8	13.8	0.77	0.54	0.039
	17–18	♀	35	55	20	59	86	27	733	480	36	0.67	20.7	13.7	0.85	0.57	0.042
XV	77–78	♂	30	50	20	60	102	42	752	525	36	0.70	21	15	0.74	0.51	0.035
	79–80	♀	30	50	20	60	99	33	803	546	35	0.68	23	16	0.81	0.55	0.035

from consideration of a chemical analysis of the rat at the age of those in the experiment, assuming a growth of 2 gm. a day and a consumption of 10 gm. of food and providing for an excess of these three elements.

The compositions of Diets I and II are given in Tables II and III, and the composition of the ash in Table IV.

TABLE II.
Diets.

	Diet No.	Stock mixture.	Salt mixture.	Vitamins.	
				Spinach extract.	Harris yeast extract.
		gm.	*gm.*	*per cent*	*gm.*
Controls.	I	97	3.0	20	0.2
	II	98	2.075	5	0.25
	XV	97	2.75	5	0.25

TABLE III.
Salt Mixtures.

Diet No.	NaCl	$CaCO_3$	K_2HPO_4	$MgSO_4$	$Fe_2(SO_4)_3$
	gm.	*gm.*	*gm.*	*gm.*	*gm.*
I	1.25	1.0	0.5	0.15	0.1
II	1.25	0.5	0.225	0.05	0.05
XV	1.25	1.0	0.9	0.05	0.05

TABLE IV.
Analysis of Ash of Diets.

Diet No.	Mg. per 100 gm. of diet.			Ratios.		
	Ca	P	Mg	P:Ca	Ca:Mg	P:Mg
I	390	252	25	0.65	15.6	10.1
II	205	150	16	0.73	12.8	9.4
XV	402	288	17	0.71	24.0	17.0

The data in Table I show that with increasing age there is a gradual increase in the percentage of calcium in the body and it is greater in the female than in the male. The percentage of magnesium remains approximately constant. There is no appreciable sex difference. The percentage of phosphorus increases very

TABLE V.

Metabolic Balance.

Diet No.	Rat No.	Sex.	Weight of food eaten.	Estimated mineral content of food.			Excreta.						Balance.			Balance per gm. gain in weight.		
							Ash.			Ratios.								
				Ca	P	Mg	Ca	P	Mg	P:Ca	Ca:Mg	P:Mg	Ca	P	Mg	Ca	P	Mg
			gm.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*				*mg.*	*mg.*	*mg.*	*mg.*	*mg*	*mg.*
I	9–11	♂	239	939	602	60	501	265	40	0.52	12.8	6.8	438	337	20	7.8	5.9	0.4
	12–13	♀	213	848	537	53	450	235	31	0.52	14.5	7.9	398	302	22	8.5	7.4	0.5
II	14–16	♂	256	525	384	41	164	167	24	1.01	7.0	6.9	375	217	16.7	8.6	5.0	0.4
	17–18	♀	307	629	461	49	313	320	47	1.11	6.7	6.8	258	141	3	9.7	5.3	0.1

slightly and is somewhat greater in females. The P:Ca ratio decreases with age, the Ca:Mg ratio increases, while the P:Mg ratio is nearly constant.

In Table V is recorded the metabolic balance of each rat estimated from the amount and composition of the food eaten and the analysis of the excreta. The balance per gm. of weight gained averaged 8.2 mg. of Ca, 5.5 mg. of P, and 0.4 mg. of Mg in the

TABLE VI.
Diets.

	Diet No.	Stock mixture.	Salt mixture.	Vitamins.	
				Spinach extract.	Harris yeast vitamin.
		gm.	*gm.*	*per cent*	*gm.*
Controls with inanition.	III	96	4.0		0.2
	IV	97	3.0	5	0.2

TABLE VII.
Salt Mixtures.

Diet No.	NaCl	$CaCO_3$	K_2HPO_4	$MgSO_4$	$Fe_2(SO_4)_3$
	gm.	*gm.*	*gm.*	*gm.*	*gm.*
III	2.25	1.0	0.5	0.1	0.15
IV	1.25	1.0	0.5	0.15	0.1

TABLE VIII.
Analysis of Ash of Diet.

Diet No.	Mg. per 100 gm. of diet.			Ratios.		
	Ca	P	Mg	P:Ca	Ca:Mg	P:Mg
III	425	232	22	0.55	19.3	10.6
IV	382	200	21	0.52	18.2	9.5

male, and 9.1 mg. of Ca, 6.3 mg. of P, and 0.4 mg. of Mg in the female. This agrees with the characteristic sex differences as shown in the previous tables.

Summary of Composition of Normal Rat.

In summarizing the data as to the composition of normal rats, it may be said that the percentages of calcium in the normal rat

range from 0.70 at 29 days to 0.79 at 60 days in males, and from 0.73 to 0.90 in females of the same ages. These values agree closely for those recorded by Sherman and MacLeod (1925). The percentage of phosphorus increases slightly, 0.52 to 0.53 in the male and from 0.54 to 0.55 in the female, during the same period. Buckner and Peter (1922) also gives somewhat higher figures for the female than for the male. The average percentage of magnesium remained approximately constant, varying in individual cases from 0.038 to 0.051 per cent.

The P:Ca ratio decreased with age. This agrees with the findings of Hammett (1925) for the composition of the humerus and femur of the albino rat. The Ca:Mg ratio increases with age.

The male rats gained on an average 2.6 gm. daily, and the female 1.7 gm. in body weight. This is without any antirachitic vitamin for a period of 20 days. The food and the rats were shielded from ultra-violet rays. The rats did not show any signs of rickets.

In the experiment with high calcium feeding the intestines of the rats were removed before ashing in order to avoid the error which might result from the presence of large amounts of unabsorbed calcium. The control rats were also ashed with intestines removed. The diet of the latter was listed in Tables II to IV as No. XV, and the diet of those on high calcium in Tables XI to XIII as No. XVI. The composition of the ash of the control rats is given in Table I, and of the experimental ones, in Table XIV.

As may be seen from these tables, the percentage of calcium in the rat increased considerably on a high calcium diet, the controls containing 0.74 per cent (♂) and 0.81 per cent (♀) as compared with 0.86 per cent (♂) and 0.96 per cent (♀) in the experimental rats. This storing of calcium must have been entirely in the bones and intestinal contents, since Corley and Denis (1925) report that the feeding of calcium salts to rabbits does not increase the calcium content of their tissues. In these rats, phosphorus was also slightly increased and magnesium decreased.

Effects of Inanition.

Some of the control rats did not eat adequate amounts of the food and hence did not grow normally, probably because of defici-

TABLE IX.

Composition of Rat.

Diet No.	Rat No.	Sex.	Age at beginning of experiment.	Age at close of experiment.	Days on experiment.	Weight at beginning of experiment.	Weight at close of experiment.	Gain in weight.	Ash of rat.								
									Ca	P	Mg	Ratios.			Per cent of weight of rat.		
												P:Ca	Ca:Mg	P:Mg	Ca	P	Mg
			days	*days*		*gm.*	*gm.*	*gm.*	*mg.*	*mg.*	*mg.*						
III	19–21	♂	29	42	13	55	53	2	468	358	23	0.77	20.6	15.8	0.88	0.67	0.042
	22–24	♀	29	42	13	47	47	0	445	354	22	0.79	20.1	15.8	0.95	0.75	0.048
IV	25	♀	27	48	21	38	58	20	503	302	29	0.60	17.4	10.4	0.87	0.52	0.050

TABLE X.

Metabolic Balance.

Diet No.	Rat No.	Sex.	Weight of food eaten.	Estimated mineral content of food.			Excreta.						Balance.			Balance per gm. gain in weight.		
							Ash.			Ratios.								
				Ca	P	Mg	Ca	P	Mg	P:Ca	Ca:Mg	P:Mg	Ca	P	Mg	Ca	P	Mg
			gm.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*				*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
III	19–21	♂	103	437	273	23	364	219	23	0.60	14.9	9.5	73	26	0.33	13.3	5.0	0.15
	22–24	♀	91	388	212	20	289	141	20	0.53	13.8	6.7	102	71	0.33	22.4	16.8	0.50
IV	25	♀	78	680	356	37	457	251	24	0.56	15.2	8.56	223	99	13	11.2	5.0	0.7

ency of the diets in one or more vitamins or because of lack of appetite. Tables VI to VIII give the compositions and analyses of the diets and Tables IX and X the compositions of the rats and their metabolic balances.

Rats on Diet III refused to eat sufficient food and died of inanition in from 7 to 19 days. All but two weighed less at the close of the experiment than at the beginning; nevertheless, these rats gained calcium and phosphorus, as shown by their metabolic balances, while magnesium remained approximately constant. The percentages of calcium and phosphorus in their bodies was therefore slightly higher than for normal rats of their weights and the percentages of magnesium about normal. For rats of their own age, however (42 days), they contained low calcium, phosphorus, and magnesium (see Table I). Females stored more of each element per gm. of gain in weight than did males.

One rat on Diet IV also failed to grow normally. For rats of its weight, its percentage composition was about normal.

Summary of Inanition.

Rats in inanition grew less than normal rats or lost weight. Nevertheless, they stored calcium and phosphorus so that the percentages of these in the body tended to increase. The actual amount of each was less than for normal rats of their age. The percentage of magnesium remained constant, and the percentages of calcium and phosphorus are much nearer normal than the total calcium and phosphorus compared with normal rats of the same age. Therefore, in later experiments in which the rats did not grow at the normal rate, the percentages should be compared and not the totals on the basis of age.

Effects of Varying the Mineral Ratios.

In the seven experiments given in Tables XII to XV (Diets V to XI), variations in the P:Ca, Ca:Mg, and P:Mg ratios were employed to determine any possible effects upon the compositions of the rats.

On a low calcium diet (Diet V) there was practically no growth,

TABLE XI.

Diets.

	Diet No.	Stock mixture.	Salt mixture.	Vitamins.	
				Spinach extract.	Harris yeast vitamin.
		gm.	gm.	per cent	gm.
Low Ca.	V	98	2.25	5	0.25
High P.	VI	97	2.85	5	0.25
" "	VII	96.5	3.50	20	0.20
" "	VIII	96	3.95	5	0.25
Low P.	IX	97	2.70	10	0.20
High Mg.	X	97	3.13	10	0.20
Low "	XI	97	3.025	10	0.20
High Ca.	XVI	95	4.85	5	0.25

TABLE XII.

Salt Mixtures.

Diet No.	NaCl	$CaCO_3$	K_2HPO_4	$MgSO_4$	$Fe_2(SO_4)_3$	KCl	$MgCO_3$	Na_2SO_4
	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.
V	1.25		0.9	0.05	0.05			
VI	1.25	0.6	0.9	0.05	0.05			
VII	1.25	0.75	1.25	0.15	0.1			
VIII	1.25	0.6	2.00	0.05	0.05			
IX	1.25	1.0		0.15	0.1	0.2		
X	1.25	1.0	0.5	0.175	0.1		0.105	
XI	1.25	1.0	0.5		0.1			0.175
XVI	1.25	2.6	0.9	0.05	0.05			

TABLE XIII.

Analysis of Ash of Diets.

	Diet No.	Mg. per 100 gm. of diet.			Ratios.		
		Ca	P	Mg	P:Ca	Ca:Mg	P:Mg
Low Ca.	V	122	298	15	14.9	1.5	19.9
High P.	VI	244	272	15	1.11	16.3	18.1
" "	VII	330	337	24	1.02	13.8	14.1
" "	VIII	250	468	15	1.87	16.7	31.5
Low P.	IX	372	124	20	0.34	18.6	6.2
High Mg.	X	390	250	41	0.64	9.7	6.1
Low "	XI	400	250	6	0.63	66.7	14.7
High Ca.	XVI	1018	281	15	0.28	67.8	18.7

TABLE XIV.

TABLE XIV.

Composition of Rat.

Diet No.	Rat No.	Sex.	Age at beginning of experiment.	Age at close of experiment.	Days on experiment.	Weight at beginning of experiment.	Weight at close of experiment.	Gain in weight.	Ash of rat.								
									Ca	P	Mg	Ratios.			Per cent of weight of rat.		
												P:Ca	Ca:Mg	P:Mg	Ca	P	Mg
			days	*days*		*gm.*	*gm.*	*gm.*	*mg.*	*mg.*	*mg.*						
V	26–28	♂	36	49	20	67	70	3	505	331	29	66	17.6	11.6	0.73	0.47	0.041
	29–31	♀	31	67	20	50	51	1	389	265	24	68	16.9	11.5	0.77	0.53	0.046
VI	32–34	♂	36	56	20	67	86	19	759	466	35	61	21.6	13.3	0.88	0.54	0.041
	35–37	♀	31	51	20	47	61	14	520	323	29	62	18.1	11.4	0.86	0.53	0.049
VII	38–40	♂	42	60	18	83	87	5	761	601	32	0.78	24.0	18.7	0.88	0.68	0.037
VIII	41–43	♂	33	53	20	60	75	15	595	397	32	0.68	18.8	12.5	0.79	0.53	0.042
	44–46	♀	36	56	20	60	77	17	646	402	36	0.62	18.6	11.4	0.84	0.52	0.045
IX	47–49	♂	28	48	20	45	75	30	454	268	21	0.61	21.5	12.8	0.60	0.36	0.028
	50–52	♀	41	59	18	76	87	11	754	410	27	0.56	26.9	14.7	0.80	0.46	0.030
X	53–55	♂	37	57	20	76	102	27	824	671	48	0.82	17.1	14.0	0.80	0.66	0.047
	56–57	♀	37	57	20	64	97	33	874	612	48	0.70	18.6	13.0	0.90	0.63	0.049
XI	58–59	♂	37	57	20	71	120	49	1099	687	38	0.62	29.4	19.0	0.92	0.67	0.032
	60–62	♀	37	57	20	69	92	23	894	617	26	0.69	35.2	24.2	0.98	0.63	0.028
XVI	81–83	♂	30	50	20	63	116	53	993	646	33	0.65	30	20	0.86	0.56	0.028
	84–86	♀	30	50	20	65	105	40	1012	647	34	0.64	31	19	0.96	0.62	0.032

TABLE XV.

Metabolic Balance.

Diet No.	Rat No.	Sex.	Weight of food eaten.	Estimated mineral content of food.			Excreta.						Balance.			Balance per gm. gain in weight.		
							Ash.			Ratios.								
				Ca	P	Mg	Ca	P	Mg	P:Ca	Ca:Mg	P:Mg	Ca	P	Mg	Ca	P	Mg
			gm.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*				*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
V	26-28	♂	161	36	481	24	42	472	23	11.62	2.1	24.0	−6	9	1	−4.5	11.8	0.1
	29-31	♀	163	36	484	24	42	504	28	12.20	1.6	18.5	−6	13	1	−5.6	−18.0	1.8
VI	32-34	♂	185	452	504	28	232	413	17	1.85	14.1	26.2	220	91	11	11.4	4.7	0.6
	35-37	♀	193	472	526	29	311	457	19	1.47	16.9	25.0	161	69	10	11.8	4.9	0.5
VII	38-40	♂	187	616	629	45	489	500	42	1.07	10.1	10.4	135	129	4	8.7	10.2	0.4
VIII	41-43	♂	145	362	674	25	204	595	20	2.88	10.4	30.1	154	78	7.3	10.2	5.3	1.0
	44-46	♀	156	389	504	25	229	622	21	2.76	11.2	30.4	160	105	8.5	9.7	6.6	1.2
IX	47-49	♂	256	952	317	51	788	298	48	0.38	16.4	6.2	164	19	3.0	5.5	0.6	0.1
	50-52	♀	171	634	212	34	505	239	34	0.53	16.6	8.2	129	−27	0.3	−57.3	15.6	1.7
X	53-55	♂	184	717	461	75	434	219	59	0.51	7.7	3.7	280	242	16	10.6	9.6	0.6
	56-57	♀	220	856	549	90	469	327	65	0.70	7.3	5.0	388	273	26	11.7	8.6	0.8
XI	58-59	♂	222	886	554	13	294	252	12	0.86	25.6	22.0	593	302	2	12.2	6.3	0.03
	60-62	♀	202	807	505	12	400	295	12	0.74	33.8	24.7	406	210	0	18.1	8.4	0.03

the average daily increase in weight being 0.15 gm. for the males and 0.05 gm. for the females. There was an absolute decrease in the total calcium content of each rat, so that their percentage composition in calcium was less at the close than at the beginning of the experiment. The absolute content of phosphorus increased

TABLE XVI.
Diets.

	Diet No.	Stock mixture.	Salt mixture.	Vitamins.	
				Spinach extract.	Harris yeast vitamin.
		gm.	*gm.*	*per cent*	*gm.*
Low Mg.	XII	96.5	3.775	10	0.2
" Ca.	XIII	97.0	3.0		0.2
" Mg.	XIV	96.0	4.025		0.2

TABLE XVII.
Salt Mixture.

Diet No.	NaCl	$CaCO_3$	K_2HPO_4	$MgSO_4$	$Fe_2(SO_4)_3$	Na_2SO_4
	gm.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
XII	1.25	1.0	1.25		0.1	0.175
XIII	2.25		0.5	0.15	0.1	
XIV	2.25	1.0	0.5		0.1	0.175

TABLE XVIII.
Analysis of Ash of Diets.

Diet No.	Mg. per 100 gm. of diet.			Ratios.		
	Ca	P	Mg	P:Ca	Ca:Mg	P:Mg
XII	322	325	7	1.01	46.0	46.4
XIII	54	240	25	4.44	23.0	9.6
XIV	410	235	7	0.61	58.6	33.6

slightly on the average (not in all individuals) and magnesium remained approximately constant. Males and females were about equally affected.

In Diets VI, VII, and VIII the P:Ca ratio was increased from the normal of about 0.73 in the control diets to 1.87 in Diet VIII. In Diet VII both calcium and phosphorus were increased. In all

TABLE XIX.

Composition of Rat.

Diet No.	Rat No.	Sex.	Age at beginning of experiment.	Age at close of experiment.	Days on experiment.	Weight at beginning of experiment.	Weight at close of experiment.	Gain in weight.	Ash of rat.								
									Ca	P	Mg	Ratios.			Per cent of weight of rat.		
												P:Ca	Ca:Mg	P:Mg	Ca	P	Mg
			days	*days*		*gm.*	*gm.*	*gm.*	*mg.*	*mg.*	*mg.*						
XII	63–64	♂	42	67	25	80	80	1	714	521	26	0.73	27.5	20.0	0.89	0.65	0.033
XIII	65–67	♂	29	51	22	54	65	5	381	313	23	0.83	16.5	13.6	0.67	0.55	0.041
	68–70	♀	29	89	66	53	69	16	444	371	27	0.83	16.1	13.6	0.67	0.56	0.042
XIV	71–73	♂	32	44	8	69	66	−3	579	406	25	0.71	23.9	17.1	0.90	0.62	0.038
	74–76	♀	32	39	7	56	49	−7	460	369	21	0.80	21.9	17.7	0.94	0.75	0.042

TABLE XX.

Metabolic Balance.

Diet No.	Rat No.	Sex.	Weight of food eaten.	Estimated mineral content of food.			Excreta.						Balance.			Balance per gm. gain in weight.		
							Ash.			Ratios.								
				Ca	P	Mg	Ca	P	Mg	P:Ca	Ca:Mg	P:Mg	Ca	P	Mg	Ca	P	Mg
			gm.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*				*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
XII	63–64	♂	289	931	940	20	786	884	30	1.13	26.2	29.4	145	57	−10	145	57	−10
XIII	65–67	♂	163	88	395	41	81	393	40	4.98	2.0	9.1	7.0	−26	1	0.5	0.2	0.3
	68–70	♀	356	226	854	93	216	819	92	4.07	2.4	8.8	−17.0	−37	−3	0.6	−3.0	−0.4
XIV	71–73	♂	103	424	247	7	362	223	14	0.61	25.0	15.8	62	49	−7	−20.7	−9.9	2.3
	74–76	♀	52	215	126	4	158	96	10	0.63	13.1	9.6	57	29	−6	−5.7	−3.3	0.4

the rats the normal ratios of calcium, phosphorus, and calcium were maintained, indicating that the normal composition of the rat (mg. per gm. of weight) cannot be altered by increasing the amount of phosphorus in the diet above that needed for normal growth. Altering the P:Ca ratio does not have any effect as long as both are present in adequate amounts, and the calcium is not present in massive doses.

The low P diets produced typical rickets which was more severe the more rapid the growth.

Two of the rats (Nos. 49 and 50) on this diet refused to eat and therefore lost weight. Rat 50 died after 14 days. The other two, Rats 51 and 52, grew somewhat less than normal rats (about 1.5 gm. daily). In respect to calcium the two former showed the typical effects of fasting (high percentage of calcium), while the two latter had a greatly decreased calcium content. Rat 51, which grew most rapidly, had slightly more than one-half the normal amount of calcium for its weight. The two rats which fasted lost phosphorus, one of the others lost phosphorus, and one gained slightly. At the close of the experiment, the percentage of phosphorus in the two rats which grew was about half that of normal rats of about the same weight (0.27 and 0.36 per cent as compared with about 0.60 per cent in the normal rat). In the rats which fasted, the percentage was higher, but less than for normal rats (combined effects of fasting and low phosphorus). The percentage of magnesium was low.

On a high magnesium diet (Diet X) the rats were normal in respect to percentage of calcium and phosphorus, but contained on the average an increased percentage of magnesium. The percentage of magnesium did not rise, however, above the upper limits found in normal rats.

Diet XI was low in magnesium. At the close of the experiment the percentage of magnesium in the ash of the rat was slightly reduced below the average, but not below the low limit of that found in the control rats. The magnesium content of the diet was approximately 6 mg. for each 100 gm. of diet, which means that assuming an average intake of 10 gm. of food daily, each rat received 0.6 mg. of magnesium daily. Allowing for a daily growth of 2.8 gm. (♂), it may be estimated that each male rat needs about 1.1 mg. daily for normal growth. The female should receive about

0.7 mg. daily. The rats on this experiment grew at approximately normal rates (males slightly faster, females slightly slower), hence the males could not have received enough magnesium for normal growth even though all was absorbed from the alimentary tract. The percentage of calcium was increased in these rats. This effect cannot be ascribed to fasting since growth was approximately normal.

Inanition.

In the experiments recorded in Tables XVI to XX the rats on the experimental diets refused to eat adequate amounts and hence failed to grow normally.

In Diet XII phosphorus was increased slightly, calcium was present in about normal amounts, while the magnesium content was low. Two rats were kept on this diet for 25 days. They refused to eat the food and failed to grow. Their balances of calcium and phosphorus were positive, while that of magnesium was negative. Upon analysis they showed a high phosphorus and calcium percentage, and low percentage of magnesium.

When inanition is combined with calcium starvation (Diet XIII) the percentage of calcium in the body decreases, in contrast to the increase which occurs during inanition in the presence of adequate amounts of calcium. The percentage of phosphorus also fails to increase and remains approximately normal. The percentages of calcium, phosphorus, and magnesium in these rats approach much more nearly those of the control rat than do the percentages in a rat in simple inanition.

The rats on Diet XIV also fasted and failed to grow. All lost weight or remained stationary. In these rats the percentages of calcium and phosphorus were above normal, while magnesium was approximately normal, in spite of a slight negative magnesium balance.

Rickets.

X-ray photographs taken after the rats were killed showed only one group with discernible abnormalities of bone structure—the rats with low phosphorus in their diets (120 mg. per 100 gm. of diet) had severe rickets. Pappenheimer, McCann, and Zucker (1922) state that when phosphates (calculated as phosphorus) are

reduced to 135 mg. per 100 gm. of diet, rickets begins to appear and is active when phosphorus is present only to the amount of 110 mg. per 100 gm. of diet. As stated above, the absolute amount of phosphorus and not the P:Ca ratio seems to be the determining factor.

Those rats receiving low calcium did not show any signs of rickets nor did those with the phosphorus content of the diet at a higher level than that of calcium. Rickets of this type has been described by Pappenheimer, McCann, and Zucker (1922) and McCollum and Simmonds *et al.* (1922). Cramer states that rickets appears when the phosphorus per 100 gm. of diet is increased to 1 gm. and the calcium is decreased to 22 mg. No ratio similar to this was employed in these experiments.

SUMMARY.

1. Normal rats from one litter were found to contain at 29 days of age 0.70 per cent calcium, 0.52 per cent phosphorus, 0.045 per cent magnesium (♂), and 0.75 per cent calcium, 0.54 per cent phosphorus, and 0.045 per cent magnesium (♀). At 60 days, the percentages were about 0.79 for calcium, 0.53 for phosphorus, and 0.045 for magnesium (♂), and 0.90 for calcium, 0.55 for phosphorus, and 0.045 for magnesium (♀).

These figures agree closely with averages read from a smoothed curve. According to such a curve, a male rat of 48 gm. (29 days) in weight contains 0.70 per cent calcium, 0.53 per cent phosphorus, and 0.046 per cent magnesium, and a female of 36 gm. (29 days) contains 0.74 per cent calcium, 0.53 per cent phosphorus, and 0.046 per cent magnesium. From the same curve a male rat of 135 gm. (57 days) contains 0.79 per cent calcium, 0.58 per cent phosphorus, and 0.38 per cent magnesium. A female of 122 gm. (57 days) contains 0.84 per cent calcium, 0.61 per cent phosphorus, and 0.39 per cent magnesium.

2. In inanition growth is decreased and the percentage composition of calcium and phosphorus tends to increase. The percentage of magnesium remains approximately constant.

3. When the percentage of calcium in the diet is decreased, growth is inhibited. An absolute decrease in calcium may occur, so that the percentage of calcium in the rat is considerably decreased. Phosphorus is unaffected, and magnesium is decreased.

When calcium in the diet is increased, there is a storing of calcium in the bones in greater amounts than in normal rats. Phosphorus also is increased in the ash of the rat, and magnesium slightly decreased.

4. When phosphorus is increased in the diet rats grow normally —all three elements being present in normal amounts.

5. When phosphorus is decreased in the diet, less phosphorus is stored than in normal rats, or may even be lost, so that the percentage may be reduced to approximately one-half the usual figure. The percentages of calcium and magnesium also become reduced.

6. On a high magnesium diet the average percentage of magnesium in the body increases slightly. The other two elements are unaffected.

7. On a low magnesium diet, the average percentage of magnesium is decreased slightly. The percentages of calcium and phosphorus may increase.

8. Although the ratios of Ca, P, and Mg in the rat may be varied widely by varying the ratios in the diets, the x-ray photographs do not show any abnormalities in the rats, except those where the absolute amount of phosphorus is low (about 120 mg. per 100 gm. of diet). These rats acquired rickets.

9. The composition of the rat in respect to magnesium is more constant under varying conditions than in respect to calcium or phosphorus. In no case could a decrease or increase be obtained greater than that shown in the variations of normal rats.

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ON THE RESIDUAL REDUCTION OF BLOOD.

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For the determination of the blood sugar concentration the reduction method, on account of the exactness and rapidity with which it works even where one has to deal with very small quantities of blood, has been adopted and is most commonly used at present.

It is, therefore, of great importance to ascertain how great a portion of the reducing substance represents fermentable carbohydrates and how great a part of it is due to reducing substances of quite a different nature (such as creatine, creatinine, uric acid, etc.), the so called residual reduction.

I have previously made this question the object of critical investigations (1, 2), occupying myself chiefly with the amount of residual reduction in Bang's micro method. Together with Rasmussen, I have likewise determined the residual reduction in a few individual cases (3), where the method of Hagedorn and Norman Jensen was used, a method which is distinguished by its great facility and exactness.

The result of these investigations was that the residual reduction in Bang's micro method for normal blood varied between 0.000 and 0.008, varying as a rule, however, between 0.003 and 0.005 per cent, that is to say, between 3 and 5 per cent of the total reduction value only; this signifies that, in most cases, one can do without the residual reduction and use the reduction values as a standard for the content of fermentable carbohydrates in the blood. However, this does not apply to cases of uremia. Thus, in rabbits, a residual reduction of 0.028 and 0.035 per cent is found 3 days after the extirpation of the kidney. In other words, the residual reduction can here reach such an amount that its presence

will be able to gain importance for the evaluation of the reduction determinations.

Hiller, Linder, and Van Slyke have come to quite a different conclusion, for they find a value of 0.03 (4) for the normal residual reduction, varying from 0.01 to 0.09,

The question has now gained a special interest, because the authors maintain that this value persists unchanged after the application of insulin. The real blood sugar concentration during the state of hypoglycemic shock is therefore still lower than previously assumed; in two cases out of six, the glucose concentration, when corrected for the great residual reduction, was 0.

However, I fail to perceive that this conclusion is really justified by the paper mentioned above, for it does not appear that—contrary to what was the case with my previous experiments—due allowance has been made for the principal cause of systematic errors in the residual reduction determination, namely to an incomplete fermentation.

Hiller, Linder, and Van Slyke terminate the fermentation after the lapse of ½ hour. What errors this may lead to will best be seen by the following experiments.

To 0.1 per cent glucose in Ringer's solution is added one-tenth volume of 10 per cent yeast solution. Thus, the proportion of yeast and glucose contained in the solution is 10:1. The fermentation takes place at 30–35°C.

	Glucose. *per cent*
Original	0.100
After 1 hr.'s fermentation	0.053
" 2 hrs. "	0.029
" 3 " "	0.010
" 5 " "	0.005
" 24 " "	0.0008
" 36 " "	0.0007
" 60 " "	0.0005
" 108 " "	0.00004

In spite of the great quantity of yeast the glucose disappears only after the lapse of about 24 hours; if the fermentation had been terminated for example after 1 to 2 hours, a residual reduction of from 0.06 to 0.03 would have been found notwithstanding that the actual residual reduction here is 0.

In blood the fermentation takes place somewhat more rapidly.

In blood from which the reducing substances (glucose as well as uric acid, etc.) had been removed by means of dialysis, but to which glucose had again been added, the following reduction values were obtained with the same quantity of yeast.

	Dialyzed blood.	Ringer's solution (contains 0.1 per cent asparagine).
Original	0.10	0.10
After 2 hrs. fermentation	0.028	0.027
" 7 " "	0.008	0.014
" 19 " "	0.0025	0.004
" 41 " "	0.0008*	0.0006

* 0.0004 of which, however, was reducing particles which it has been impossible to remove by the dialysis of the blood. By employing Bang's micro method in the particular manner which I have described (*cf.* (5) p. 91) it has been possible to determine reducing substance in a quantity corresponding to 0.0002 per cent of glucose.

If, therefore, after a fermentation, a reduction for example of 0.03 per cent is found, one has, especially when the fermentation has been of so short a duration as is the case in the experiments described in the work mentioned above, no point of support for a reduction of this magnitude. The result may just as well be due to an incomplete fermentation. Reliable residual reduction determinations can only be performed if control experiments carried on simultaneously show that the yeast employed under entirely corresponding conditions has actually been able to remove quantitatively the glucose within the time employed.

It is, of course, possible to accelerate the fermentation still more by using greater quantities of yeast than those I have employed,[1] but in that case the correction, which must be used for the auto-reduction of the yeast, becomes so extensive that it appears somewhat hazardous simply to subtract this value from the residual reduction found, as particularly Neuberg (6) and Mayer (7) have pointed out. Thus, Neuberg in Der Harn (vol. i, p. 329) writes:

"Als durchaus verfehlt müssen die vielen Versuchsanordnungen gelten, wo man nach Vergärung etwa vorhandenen Zuckers etwas über die Natur

[1] Such as Hiller, Linder, and Van Slyke have done.

der reduzierenden Nichtzuckerstoffe des normalen Harnes zu eruieren bemüht war. Denn die arbeitende oder sich (bei Zuckermangel) zersetzende Hefe liefert Purine und optisch aktive Stoffe, darunter Kohlenhydrate wie Hefengummi in Lösung, die demnach mehr verunreinigt als von störenden Stoffen befreit wird."

As one has no guarantee for these substances being formed in exactly the same quantity both in the experiment itself and in the control experiment with yeast alone, it cannot be expected, as I have previously asserted ((3) p. 999), that a subtraction of the autoreduction of the yeast will lead to a sure result, unless this autoreduction be small.

In my experiments the autoreduction of the yeast in the quantity employed was about 0.0003 per cent, calculated as glucose.

Such control experiments have been carried on along with all my previous investigations; whereas the rather few control experiments with a view to the completeness of the fermentation mentioned by Hiller and coworkers (4) are not convincing.

In the experiments mentioned one sees in some cases that the reduction value, after the lapse of ½ hour, has fallen for example to 0.03 per cent, a value which is again found after an additional ½ hour's fermentation. However, it is not feasible to conclude from this fact that the fermentable carbohydrates have been destroyed. Seeger (8) as well as Lippmann (9) point out that the fermentation in pure sugar solutions can cease when the sugar concentration is lower than 0.1 to 0.03 per cent. If it then happens that the reduction value in the experiments mentioned in certain cases rises very considerably (from 0.03 to 0.04 per cent) during the final fermentation, it becomes evident that the determinations must be possessed of a systematic error, which will be able to conceal completely the slight, though steady fall of the glucose concentration which, according to my experiments, is obtained in the last part of the fermentation period, a fall which just reveals that the fermentation has not yet been brought to an end.

The rise in the reduction value is certainly due to the circumstance that the blood has been allowed to stand after it has been drawn. If the blood is allowed to stand for some time, it will often be very difficult to bring about such a precipitation that the reduction value, for that reason alone, does not become too

high. Thus, it is a well known fact that, if only a hemolysis is established, it will be difficult to bring about a precipitation of protein substances by the method of Hagedorn and Norman Jensen, and the result will be that the reduction value and, therefore, the residual reduction value, is found to be too high.

That this has been the case in the work mentioned above seems just be to verified by the fact that, in certain cases, an increase in the reduction value is found during the last fermentation period.

That I, on the other hand, have been successful in avoiding (or nearly avoiding) this error by using colloidal iron for the precipitation, is evidenced by my previously mentioned results, which show that I succeeded in lowering the reduction value in dialyzed blood down to 0.0008 per cent after a fermentation of 41 hours.

SUMMARY.

If a fermentation investigation is to prove reliable in regard to the quantity of non-fermentable substances, it is necessary to determine the autoreduction of the yeast and, above all, to control the fermentability of the yeast. With Bang's micro method the residual reduction is very small, as a rule about 0.004 per cent. The higher values arrived at by Hiller, Linder, and Van Slyke may (and probably must) be attributed to systematic errors.

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THE RESIDUAL REDUCTION OF BLOOD.

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Ege[1] considers that Hiller, Linder, and Van Slyke[2] did not offer proof that they permitted yeast to ferment the blood sugar long enough to destroy it. However, they found a minimum reduction value after 20 minutes fermentation with the yeast employed by them, and no further decrease after six times as long. Furthermore the residual reduction obtained after 20 minutes with yeast was the same, within the limits of analytical error, as that obtained after 24 to 48 hours spontaneous glycolysis of blood. Glucose added sufficient to triple the normal reducing power of the blood was completely destroyed. We believe that these controls were sufficient to show that the conditions insured removal of whatever genuine glucose was present.

We had, in preliminary experiments not reported in the previous paper,[2] also determined the rate of action of our yeast on solutions of pure glucose of concentrations similar to those found in the blood, and found the fermentation completed in 20 minutes at 38°. Ege uses a different yeast, only about one-fifteenth as much of it, and a temperature of 30–35°. The difference between the rate of fermentation obtained in his experiments on pure sugar solutions and the rate in ours may be due to these differences in conditions. We have repeated our experiments with pure glucose, and compared them with similar fermentation experiments using the amounts of yeast employed by Ege (the race of yeast could not be duplicated). In each case control experiments were performed with yeast in Ringer's solution in order to determine the reducing power of the yeast suspension and the reductions so obtained were subtracted from those obtained in the sugar solutions. The results

[1] Ege, R., *J. Biol. Chem.*, 1926, lxviii, 317.

[2] Hiller, A., Linder, G. C., and Van Slyke, D. D., *J. Biol. Chem.*, 1925, lxiv, 625.

confirm those of Hiller, Linder, and Van Slyke, in showing that no reducing substance determinable by the Hagedorn-Jensen method is left after 20 minutes under the conditions used by them. With the conditions used by Ege, as nearly as we have been able to duplicate them, about 5 hours were required for complete disappearance of reducing substance. Neither the Hagedorn-Jensen method used by us, nor the Bang method, is capable of showing less than about 0.005 per cent of glucose.

Ege's work appears to have been carefully controlled. We believe the work of Hiller, Linder, and Van Slyke was likewise. For the fact that Ege obtained residual reduction equivalent to only 3 to 5 per cent of the total reduction while we obtained 10 to 30 per cent we have no explanation to offer.

Fermentation under Conditions of Hiller, Linder, and Van Slyke.[2]—A yeast suspension was prepared by mixing 3.2 gm. of fresh Fleischmann's compressed yeast (= 1.2 gm. of dry substance) with 5 cc. of water. The suspension contained accordingly $\frac{100 \times 1.2}{8.2} = 14.6$ per cent of dry yeast. Of this suspension 1 cc. was added to 2 cc. of Ringer's solution containing 0.1 per cent of glucose. The mixture was incubated at 37°. The reducing power was determined at intervals by the Hagedorn-Jensen method. The relatively large amount of yeast was originally used in blood experiments in order to obtain a quick fermentation, uncomplicated by changes due to bacteria or blood enzymes. The dry yeast content in the final mixture was 4.9 per cent.

Fermentation Approximating Conditions of Ege.[1]—0.5 cc. of a 10 per cent suspension of the same yeast was added to 5 cc. of 0.1 per cent glucose in Ringer's solution. The dry yeast content of the final mixture was 0.34 per cent. The mixture was incubated and analyzed as above.

The results are given in the accompanying table.

Fermentation time at 37°.	Glucose left under Ege's condition.			Glucose left under conditions of Hiller, Linder, and Van Slyke.		
	Total.	Control with yeast alone.	Difference = reduction due to glucose.	Total.	Control with yeast alone.	Difference = reduction due to glucose.
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
0			0.100			0.100
20 min.				0.053	0.054	0
hrs.						
1	0.037	0.022	0.015	0.058	0.058	0
2	0.027	0.019	0.008			
5	0.025	0.021	0.004			
24	0.025	0.025	0			

THE EFFECT OF LONG CONTINUED ADMINISTRATION OF PARATHYROID EXTRACT UPON THE EXCRETION OF PHOSPHORUS AND CALCIUM.

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In a previous publication (1), we have reported on the effect of the administration of a potent parathyroid extract (Collip) upon the excretion of nitrogen, phosphorus, calcium, and magnesium. It was found that the excretion of phosphorus and calcium was greatly increased. As a result of these experiments and of others upon thyroparathyroidectomized dogs (2), a hypothesis concerning the functioning of the parathyroid glands was formulated and presented (3).

In the experiments with parathyroid extract to which reference has been made, the doses administered were rather large, the animals were made quite sick, and two of the three died. It might be objected, therefore, that the increased excretion of phosphorus and calcium observed was due to the severe intoxication produced and that it is not to be taken as an indication of the normal functioning of the parathyroid glands. For this reason, it was considered advisable to conduct a long continued experiment, in which the conditions might more closely resemble those to be expected in clinical cases, if such exist, of hyperfunction of the parathyroids.

EXPERIMENTAL.

An adult, female dog, weighing 6.8 kilos, was placed upon a diet of 14 gm. of dried beef heart, 14 gm. of cracker meal, 14 cc. of maize oil, 9 gm. of infusorial earth, and 300 cc. of water. This furnished 1.84 gm. of nitrogen, 0.140 gm. of phosphorus, and

0.133 gm. of calcium. (This was more calcium than it had been intended to feed, but the high calcium content of this particular sample of infusorial earth was not detected until after the close of the experiment.) After several days upon this diet the collection of urine and feces was begun. After a control period of 1 week, parathyroid extract was administered. The extract was obtained from Dr. J. B. Collip, to whom we are greatly indebted for his generous help. The extract contained 10 units per cc. in April. It had been kept in the ice chest since then and had, evidently, retained a large part of its original potency. It was desired to administer as much extract as was possible without making the animal severely ill. At first, 1 cc. was injected twice each day but, as this was found to produce loss of appetite, only one dose per day was administered. Whenever the dog failed to eat all of its food promptly, the administration of the extract was omitted on the following day. Except in a few instances, all the food prepared was eaten at some time during the day. When not, it was eaten on the following day, together with the 2nd day's ration. After about 6 weeks, either because of greater tolerance or because the extract had deteriorated, the dosage could be increased to two daily injections of 1 cc. each.

The 52 days of parathyroid administration were followed by five periods of 3 days each, in which no extract was administered. Extract B, prepared in this laboratory, was then used but was found to be ineffective. In neither this dog nor in another did it produce an increase in the concentration of calcium in the serum. Another preparation, C, also obtained from Dr. Collip, was then employed. This was stated to contain about 25 units per cc. but produced no more marked effect than did the weaker extract originally employed. After administering this extract for 9 days, the animal was kept under observation for 12 additional days, during which time no extract was given.

It had been hoped that this prolonged treatment with parathyroid extract would have produced very marked changes in the bones. Radiographic examination failed to show this. Animals were badly needed for other work and this dog was so used without further study of the bones.

The twenty-one periods listed in Table I have been divided into five groups, one experimental group of 55 consecutive days,

another of 16 consecutive days, and three groups of control periods, at the beginning of the experiment, between the two experimental periods, and at the end of the entire experiment. Because it has previously been shown (1) that the effect of parathyroid extract persists for a few days after its administration has ceased and because the feces voided by a dog on any given day are largely derived from the metabolism of the previous day, the two experimental groups each include the first 3 days after the discontinuance of the parathyroid administration.

The analytical methods employed were those described in previous publications (1, 2). The data obtained are summarized in Table I, which is nearly self-explanatory. However, the figures for the daily excretion of phosphorus and calcium in the feces require some comment. Since the animal defecated irregularly, these figures could not be obtained by dividing the values for the total excretion during the several periods by the number of days in each period. Instead, the amounts of phosphorus and of calcium per gm. of silica in the feces of each period were calculated. The total amount of silica in the feces of the 99 days of the entire experiment was 695.0 gm., an average of 7.02 gm., per day. Since the amount of infusorial earth fed was constant, it seemed justifiable to calculate the daily excretion of phosphorus and calcium in the feces of the several periods by multiplying the amount of phosphorus and of calcium, respectively, per gm. of silica by 7.02. The figures so obtained are given in Columns 13 and 14 of the table.

DISCUSSION.

In the first, collected, experimental period of 55 days, the administration of parathyroid extract had only a slight effect upon the excretion of nitrogen, the value for the average daily excretion being almost exactly the same as that for the excretion during the fore period and but very little higher than that in the following 6 days. Calculating from the average amount of nitrogen excreted during the control periods (Periods 1, 11, 12), the increase during the 55 days was only 2.66 gm., or 2.5 per cent.

However, the amounts of phosphorus and of calcium in the urine were increased by 1.243 gm. and 1.538 gm., respectively, above those calculated from the amount excreted during the

TABLE I.

Effect of the Administration of Parathyroid Extract upon the Excretion of Nitrogen, Phosphorus, and Calcium.

Dog 35, female, weight 6.8 kilos at beginning and 4.8 kilos at end of the experiment. Fed daily a mixture of 14 gm. of dried beef heart, 14 gm. of cracker meal, 14 cc. of maize oil, 9 gm. of infusorial earth, and 300 cc. of H_2O, furnishing 1.84 gm. of nitrogen, 0.140 gm. of phosphorus, and 0.133 gm. of calcium.

Period.			Urine.						Feces.					Remarks.
			Total.			Per day.			Total.			Per day.*		
No. (1)	Date. (2)	Duration. (3)	Nitrogen. (4)	Phosphorus. (5)	Calcium. (6)	Nitrogen. (7)	Phosphorus. (8)	Calcium. (9)	Silica. (10)	Phosphorus. (11)	Calcium. (12)	Phosphorus. (13)	Calcium. (14)	(15)
	1925	*days*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	
1	July 27–Aug. 2	7	13.82	0.694	0.0020	1.97	0.099	0.0003	47.5	0.462	1.055	0.0684	0.1559	Control period.
2	Aug. 3– 9	7	13.43	1.066	0.276	1.92	0.152	0.0394	42.0	0.439	1.107	0.0719	0.1810	1 cc. of Extract A twice daily on 5 days, once daily on 2 days.
3	" 10–16	7	13.28	0.744	0.241	1.90	0.106	0.0344	46.4	0.495	1.160	0.0750	0.1755	1 cc. of Extract A twice on 1st day, none on 2nd, once on each of others.
4	" 17–22	6	11.10	0.808	0.151	1.85	0.135	0.0252	36.8	0.358	0.0703	0.0683	0.1344	1 cc. of Extract A once each day.
5	" 23–28	6	11.63	0.875	0.225	1.94	0.146	0.0375	39.1	0.432	0.922	0.0766	0.1653	1 cc. of Extract A daily except on 3rd and 6th days.
6	Aug. 29–	6	14.86	0.889	0.179	2.48	0.150	0.0300	51.7	0.523	0.913	0.0711	0.1240	1 cc. of Extract A daily

6	Aug. 29–Sept. 3	6	14.86	0.889	0.179	2.48	0.150	0.0300	51.7	0.523	0.913	0.0711	0.1240	1 cc. of Extract A daily except on 2nd day.
7	" 4–10	7	12.93	0.798	0.201	1.85	0.114	0.0287	38.5	0.393	0.794	0.0718	0.1446	1 cc. of Extract A daily except on 4th day.
8	" 11–17	7	13.31	0.551	0.151	1.90	0.079	0.0216	60.7	0.602	1.234	0.0696	0.1430	1 cc. of Extract A daily except on 2nd day.
9	" 18–23	6	12.72	0.664	0.110	2.12	0.111	0.0182	44.3	0.479	0.968	0.0760	0.1529	1 cc. of Extract A twice daily. Serum Ca 3 hrs. after injection on 5th day 14.1 mg. per 100 cc.
10	" 24–26	3	6.10	0.293	0.0170	2.03	0.098	0.0057	15.0	0.171	0.332	0.0800	0.1553	No extract administered.
2–10	Aug. 3–Sept. 26	55	109.36	6.688	1.551	1.988	0.1216	0.0282	374.5	3.892	8.133	0.07293	0.1524	
11	Sept. 27–29	3	5.63	0.271	0.0002	1.88	0.090	0.0001	29.9	0.294	0.573	0.0691	0.1346	Control period.
12	" 30–Oct. 2	3	5.76	0.322	0.0008	1.92	0.107	0.0003	16.2	0.167	0.327	0.0723	0.1418	" "
13	" 3– 5	3	6.35	0.360	0.0005	2.12	0.120	0.0002	27.5	0.273	0.568	0.0698	0.1453	" "
14	" 6– 8	3	6.92	0.396	0.0004	2.31	0.132	0.0001	21.3	0.208	0.414	0.0686	0.1368	" "
11–14	Sept. 27–Oct. 8	12	24.66	1.349	0.0019	2.055	0.0843	0.0001	94.9	0.942	1.882	0.06975	0.1395	
15	Oct. 9–12	4	9.79	0.538	0.0009	2.45	0.135	0.0002	27.1	0.273	0.562	0.0706	0.1452	2 doses of Extract B on 1st day, one on 2nd. Serum Ca 4 hrs. later 9.33 mg. per 100 cc. Extract inactive on other animals also.

TABLE I—*Concluded.*

Period.			Urine.						Feces.					Remarks.
			Total.			Per day.			Total.			Per day.*		
No. (1)	Date. (2)	Duration. (3)	Nitrogen. (4)	Phosphorus. (5)	Calcium. (6)	Nitrogen. (7)	Phosphorus. (8)	Calcium. (9)	Silica. (10)	Phosphorus. (11)	Calcium. (12)	Phosphorus. (13)	Calcium. (14)	(15)
	1925	*days*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	
16	Oct. 13–16	4	9.38	0.764	0.162	2.35	0.191	0.0405	21.6	0.213	0.443	0.0693	0.1440	1 cc. of Extract C twice daily on 2 days, three times daily on 2 days. Serum Ca 4 hrs. after on 4th day, 15.6 mg. per 100 cc.
17	" 17–21	5	15.27	1.028	0.324	3.05	0.206	0.0649	41.3	0.515	1.095	0.0875	0.1860	1 cc. of Extract C twice daily.
18	" 22–24	3	7.26	0.308	0.0907	2.42	0.103	0.0302	19.1	0.231	0.450	0.0852	0.1656	No extract administered.
15–18	Oct. 9–24	16	41.70	2.638	0.5776	2.606	0.1649	0.0361	109.1	1.232	2.550	0.07927	0.1641	
19	Oct. 25–27	3	6.04	0.357	0.0230	2.01	0.119	0.0077	19.2	0.191	0.386	0.0698	0.1411	Control period.
20	" 28–30	3	5.41	0.343	0.0169	1.80	0.114	0.0056	29.9	0.298	0.590	0.0700	0.1385	" "
21	" 30–Nov. 2	3	5.45	0.331	0.0143	1.82	0.110	0.0048	19.9	0.211	0.433	0.0744	0.1530	" "
19–21	Oct. 25–Nov. 2	9	16.90	1.031	0.0542	1.878	0.1146	0.0060	69.0	0.700	1.409	0.0712	0.1442	
1–21		99	206.44	12.40	2.186				695.0	7.227	15.029			

* Calculated by multiplying the average for the amount of silica excreted per day, 7.02 gm., by the amounts of phosphorus and calcium, respectively, per gm. of silica in each period.

same control periods. The increase in the fecal excretion was much less. From the data for the silica, phosphorus, and calcium excretion during Periods 1, 11, and 12, the "normal" fecal excretion of phosphorus and calcium, during the 55 days of the experimental period, may be calculated as 3.807 gm. and 8.063 gm., respectively. The figures given in the table for the excretion during the experimental period must be corrected for the amount of feces carried over from the preceding, and into the following periods. By multiplying 55 by 7.02 gm., the amount of silica per day during the entire 99 days, we obtain 386 gm. as the corrected amount of silica in the feces of this experimental period. Actually only 374.5 gm. were obtained, so that the corrected totals for the amounts of phosphorus and calcium excreted are to be found by multiplying the observed quantities by $\frac{386.0}{374.5}$. The values so obtained are 4.011 gm. of phosphorus and 8.383 gm. of calcium. These represent an increase over the normal values of 0.204 gm. and 0.323 gm., respectively. The total increase, in both urine and feces, was 1.447 gm. of phosphorus and 1.861 gm. of calcium. The ratio of calcium to phosphorus is not that found in bone and cannot be made to approach that closely by any reasonable allowance for increased phosphorus excretion due to the slight increase in protein metabolism. It may be, however, that the use of the averages of the amounts of calcium excreted in the feces before and after the 55 day experimental period has led to an error in the calculation of the normal excretion. If the latter be calculated from the amount in the after periods, alone, the amount of excess calcium excreted is increased by over 5 gm., without any increase in the excretion of phosphorus. Evidently, the preliminary period was not continued long enough to secure a constant calcium excretion.

The interpretation of the data obtained in the second course of parathyroid administration is complicated by the effect of a severe infection that developed in one leg. It was, apparently, this infection that caused the marked increase in the excretion of nitrogen which began in Period 13, reached its maximum in Period 17, and continued into Period 19. The urines of the seven periods contained 61.01 gm. of nitrogen, an increase of 14.66 gm. above that calculated from the excretion during control

Periods 11, 12, 20, and 21. It was this infection which was responsible for most of the loss of nitrogen and loss of body weight during the experiment. Disregarding the nitrogen of the feces and hair, the total loss in nitrogen during the 99 days was 24.28 gm. Of this amount, 15.01 gm. were lost in the 25 days from October 3 to 27.

The amount of phosphorus excreted in the urine increased from an average of 0.099 gm. per day in Periods 11 and 12 to as much as 0.206 gm. per day in Period 17. As compared with the amounts excreted during Periods 11, 12, 20, and 21, the excretion of phosphorus in the urine in Periods 15 to 18, inclusive, was increased by 0.949 gm. and that of calcium by 0.534 gm.

The amount of phosphorus in the feces obtained in the 16 days, October 9 to 24, was 1.234 gm. and that of calcium was 2.550. But these quantities were voided with only 109.1 gm. of silica. Correcting to the equivalent of 7.02×16, or 112.3 gm. of silica, these values become 1.269 gm. and 2.625 gm., respectively. Calculating from the fecal excretion during Periods 11, 12, 20, and 21, the normal excretion for these same 16 days is 1.143 gm. of phosphorus and 2.272 gm. of calcium. The differences are 0.126 gm. and 0.353 gm., respectively. The total increases in both urine and feces were 1.075 gm. of phosphorus and 0.887 gm. of calcium. The ratio is not that found in bone but, if the amount of phosphorus be decreased by the amount presumably due to the infection, as calculated from the increases in nitrogen and phosphorus observed in Periods 13 and 14, before the administration of parathyroid extract, the corrected value is 0.379 gm. of phosphorus and the ratio is more nearly that obtaining in bone.

The data presented can leave no doubt but that the administration of parathyroid extract does lead to an increased excretion of phosphorus and calcium and that this increased excretion can continue for a long period of time. The calcium must be derived from the bones. No other tissue contains sufficient calcium to have supplied the total *increased* excretion of at least 2.748 gm., to say nothing of the *continuous* loss, even during the control periods, of 0.012 gm. per day, or 1.188 gm. in the 99 days of the entire experiment. But the mechanism by which this removal is accomplished is by no means clear. One hypothesis has already been presented (3) but others are being kept in mind. For in-

stance, it is quite possible that the hypothetical calcium-dissolving substance never circulates in the blood but exists only in, or in the immediate vicinity of, the osteoblasts or other cells that may be responsible for the removal of calcium from bones. It is also possible that the parathyroid hormone liberates calcium from some non-osseous tissue and that, by some other mechanism, the calcium of the bones is drawn upon to make good the deficiency.

The dog seems to be peculiarly sensitive to the effect of the administration of parathyroid extract. Cats, rabbits, and rats are much more resistant and the latter two species tolerate relatively huge doses (4). It may be that their mechanisms for the excretion of calcium function more readily and they, therefore, never develop a dangerous hypercalcemia.

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THE EFFECT OF TEMPERATURE ON METABOLISM, PARTICULARLY THAT OF PROTEIN.

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The question of seasonal variation in metabolism has recently been further studied by Denis and Borgstrom (1). They investigated the level of protein intake as indicated by the total nitrogen excretion in the urine during warm and cold months in southern United States (New Orleans), and they compared the results with conditions in northeastern United States (Massachusetts). They state: "The results obtained furnish evidence in favor of the view that increase of temperature is accompanied by a decrease in protein intake, and that, apart from the variations due to seasonal changes, the inhabitants of the semitropical portions of this country probably consume an amount of protein considerably below the quantity reported as the average intake for the nation."

The literature on this subject is sufficiently reviewed by Greenwald (2) and will not be discussed here.

Having had occasion during the last 10 years to observe at first hand considerable quantitative data on the excretion of the ordinary urinary substances, especially by medical students in Massachusetts and western New York, the impression had been gained that the urinary nitrogen for this section of the country was appreciably less than Denis and Borgstrom report in their paper. They report "in the neighborhood of 15 to 18 gm." of urinary nitrogen per 24 hours for medical students of New England. And their similar figures for southern United States students is 10.6 gm.

In order to obtain further figures on the urinary nitrogen under conditions similar to those of Denis and Borgstrom, especially as

to the diet, so that southern and northern conditions could be sufficiently closely compared, and in order to obtain further data on any possible seasonal variation we have carried out the following experimental work, including in addition to the total nitrogen: the volume, specific gravity, ammonia nitrogen, uric acid, creatinine, and chlorides, because these might also undergo seasonal variation and the investigation would therefore give a more complete picture of the general metabolism.

EXPERIMENTAL.

Ten apparently normal men were chosen as subjects from which to obtain urines once a month for at least 1 year. Eight of these men were medical students chosen because of their reliability, interest, experience, and situation to continue the experiments for a long time. The other two subjects were from the staff of this Department; *viz.*, H. A. D., an assistant, and G. E. Y., one of the writers.

The subjects agreed to observe their customary diet and water drinking, especially during the collection periods. Only the two subjects from this department knew the exact object of the experiments. 48 hour urines were collected each month (between the 1st and 10th of the month) for 12 months (1925), including an extra preliminary month (December, 1924). These urines were calculated to the 24 hour basis. In this way greater accuracy is obtained than in 24 hour collections.

Meals were obtained at a variety of places: at home, at fraternity houses, at restaurants, and at other similar customary places. The winter and summer diets were very much alike in this respect.

The habits of the subjects in regard to muscular exercise were not far different if any during the winter and summer months, although the students, especially, changed from study to other work during June, July, August, and most of September.

Analytical Methods.—The 48 hour urines were preserved with chloroform, the addition being made at the first voiding. The entire volumes were brought to the laboratory for measurement by us. The specific gravity was taken by the hydrometer; total nitrogen by the macro Kjeldahl method of Folin and Wright (3); ammonia nitrogen by the permutit colorimetric method of Folin

and Bell (4); uric acid by the arsenophosphotungstic method of Benedict and Franke (5); creatinine by the colorimetric method of Folin (6); and chlorides by the Volhard method (7).

DISCUSSION.

Chart 1 shows the output of the total urinary nitrogen for each of the ten subjects. Rather large individual variations occur.

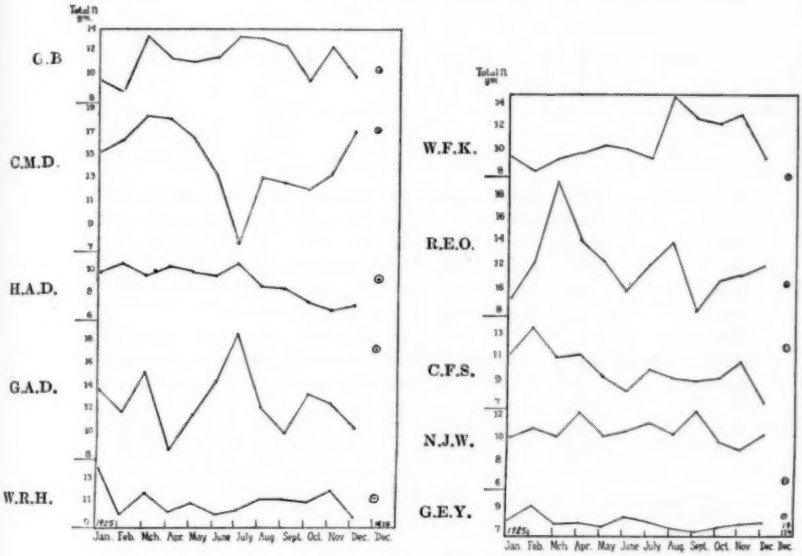

CHART 1. Total urinary nitrogen per 24 hours for ten subjects during 1925 (and December, 1924). Calculated from 48 hour collections once a month.

The average 24 hour outputs, calculated from 48 hour collections for the ten subjects are given in Table I, and curves are plotted in Chart 2. It appears that the only other constituent paralleling the volume curve rather closely is the chloride. The total nitrogen curve, however, parallels that of the volume to a rather appreciable extent. The curves for the other constituents, *viz.* ammonia nitrogen, uric acid, and creatinine, seem entirely independent of the volume, or *vice versa*.

Inspection of the total nitrogen curve shows the excretion for the year to be greatest in March, April, and August, with low periods in June, September, October, and November. For the cold and warm months there is on the whole no appreciable difference in the level of output. In reference to the other constit-

TABLE I.

Average Urinary Excretions per 24 Hours for Ten Men on Ordinary Diet. Calculated from 48 Hour Collections Once a Month.

Date.	Volume.	Specific gravity.	Total N.	Ammonia N.		Uric acid.		Creatinine.		Creatinine coefficient.*	NaCl
1925	*cc.*		*gm.*	*gm.*	*per cent of total*	*gm.*	*per cent of total N*	*gm.*	*per cent of total N*	*mg.*	*gm.*
Jan.	1226	1.022	11.231	0.402	3.63	0.434	1.29	1.691	5.88	9.1	11.91
Feb.	1081	1.023	11.321	0.532	4.86	0.385	1.16	1.820	6.16	9.8	11.33
Mar.	1334	1.020	12.709	0.494	3.96	0.449	1.26	1.869	5.77	10.1	12.16
Apr.	1305	1.019	11.578	0.393	3.37	0.443	1.26	1.575	5.30	8.5	12.61
May.	1328	1.020	11.219	0.453	4.03	0.456	1.41	1.701	5.82	9.2	12.25
June.	994	1.019	10.606	0.555	5.18	0.405	1.27	1.622	5.68	8.8	8.67
July	1224	1.015	11.187	0.445	3.98	0.477	1.42	1.641	5.45	8.9	10.15
Aug.	1450	1.014	11.602	0.570	4.76	0.428	1.23	1.682	5.39	9.1	13.72
Sept.	1333	1.017	10.507	0.429	4.16	0.478	1.54	1.606	5.86	8.7	10.29
Oct.	1300	1.017	10.465	0.442	4.31	0.483	1.57	1.479	5.47	8.0	10.00
Nov.	1098	1.017	10.869	0.493	4.48	0.569	1.78	1.593	5.63	8.6	9.49
Dec.	1151	1.017	10.145	0.310	3.02	0.520	1.79	1.604	6.16	8.7	11.18
Average	1235	1.018	11.120	0.460	4.15	0.461	1.42	1.657	5.71	9.0	11.15
Average per 70 kilo man			11.300								
1924											
Dec.	1273	1.021	11.313	0.425	3.90	0.514	1.62	1.825	6.48	9.9	13.86

* Creatinine nitrogen per kilo of body weight per 24 hours.

uents determined there is no indication of a lower metabolism in warm than in cold weather. The variation in excretion from month to month is large for some of the subjects but evidently must be explained on other seasonal variations than temperature.

That the average levels of output are not the same in successive years is shown by the levels for December, 1924, as compared

with those for December, 1925. The total nitrogen for December, 1924, is about 1.2 gm. greater than for December, 1925.

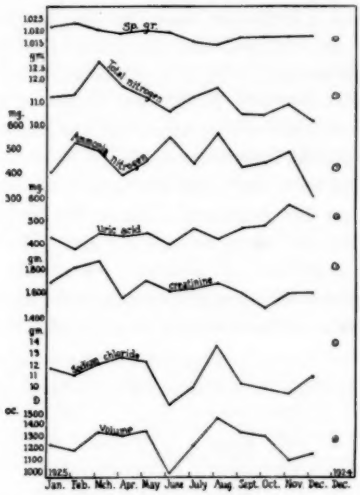

CHART 2. Average 24 hour urinary excretions for ten subjects during 1925 (and December, 1924).

TABLE II.

Average Urinary Excretions per Kilo per 24 Hours for Ten Men. Calculated from 48 Hour Collections Once a Month during 1925.

Subject.	Weight.	Volume.	Total N.	Ammonia N.	Uric acid.	Creatinine.	NaCl
	kg.	*cc.*	*gm.*	*mg.*	*mg.*	*mg.*	*gm.*
R. G. B.	65.9	22.6	0.173	10.00	6.48	24.8	0.141
C. M. D.	61.3	34.3	0.238	8.50	8.06	27.9	0.341
H. A. D.	68.1	13.5	0.135	5.89	7.37	23.1	0.142
G. A. D.	63.6	20.3	0.202	6.67	6.43	25.3	0.144
W. R. H.	80.0	11.5	0.140	4.37	7.35	23.5	0.136
W. F. K.	61.3	27.7	0.173	7.18	6.07	26.5	0.177
R. E. O.	65.9	17.4	0.187	7.95	7.00	26.7	0.139
C. F. S.	68.1	12.1	0.151	6.32	5.84	22.2	0.136
N. J. W.	68.2	15.1	0.158	4.78	7.43	24.8	0.141
G. E. Y.	86.3	10.7	0.093	4.03	4.66	19.2	0.118
Average	68.9	18.5	0.165	6.57	6.67	24.4	0.161

Table II shows the 24 hour urinary outputs per kilo of body weight for the ten subjects. One of the smallest subjects weighed 61.3 kilos, the largest, 86.3 kilos; the average for the ten subjects was 68.9 kilos, a little less than the weight usually taken for an average man, *viz.* 70 kilos (154 pounds). It appears that one of the smallest subjects, C. M. D., showed by far the most intense metabolism; subject G. E. Y., the largest in weight, showed the lowest. The output of creatinine is the most constant per kilo of any constituent determined.

Denis and Borgstrom find the average total nitrogen in the urine per 24 hours for southern United States (New Orleans) men of 70 kilo man equivalent to be 11.07 gm. We find 11.30 gm. under similar conditions for western New York men. Total nitrogen figures which we have gathered at miscellaneous times on many other subjects in this locality and in New England confirm

TABLE III.

Monthly Mean Temperatures for 1925.

Jan.	21.7	July	67.6
Feb.	30.8	Aug.	69.4
Mar.	35.8	Sept.	62.4
Apr.	44.7	Oct.	43.0
May	49.0	Nov.	38.6
June	66.0	Dec.	27.2

the above. We cannot, therefore, agree with the value of 15 to 18 gm. of urinary nitrogen per 24 hours for New England medical students, believing that the value is 35 to 65 per cent too high.

When considering the mean monthly temperatures of this locality as shown in Table III,[1] and the fact that man in temperate regions is rather easily able to adjust himself to such regional temperatures by means of variations in amount of clothing and by artificial heating facilities, it is not surprising that general metabolism, especially that of protein, should not vary much. It is probable that semitropical temperatures, however, are such as to call somewhat on the diet factor to aid the individual in maintaining himself against greater climatic variations than are found in temperate regions. This probably explains the lower

[1] We are indebted to the United States Weather Bureau at Buffalo for these data.

protein metabolism in summer than in winter which Denis and Borgstrom find.

SUMMARY.

Monthly collections of 24 hour urines for ten subjects for 1 year (1925), on an ordinary diet, have been analyzed as to volume, specific gravity, total nitrogen, ammonia nitrogen, uric acid, creatinine, and chlorides. The total nitrogen excretion is shown graphically for each subject. The averages are given for all of the constituents. The average total urinary nitrogen per 70 kilo man is 11.30 gm.

CONCLUSIONS.

Seasonal variations in temperature in temperate zones are not great enough to influence the level of protein metabolism in man; it is the same in summer as in winter.

The difference in protein metabolism between inhabitants of temperate zones (New England and western New York) and of semitropical zones (southern United States) is small and not of the magnitude indicated by Denis and Borgstrom; *viz.*, a difference of 35 to 65 per cent.

We wish to express our thanks and appreciation to the medical students who were subjects in these experiments. Without their cooperation we could not have conducted these experiments.

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CHANGES IN THE BLOOD COMPOSITION OF RABBITS FED ON RAW SOY BEANS.

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About 50 years ago Haberlandt (11) wrote that the time would come when soy beans would play a very important rôle in the diet of the poor, and that soy beans would be of much greater importance as admixture for potatoes than salt, because of their high fat and protein content. During the Great War the demand for cheaply produced and easily obtainable nutrients, particularly for suitable proteins and fat, directed attention anew to the possibilities of the soy bean. Daniels and Nichols (9) found that an economic consideration of the yellow soy bean led to the conclusion that it is one of the most valuable of the leguminous seeds. It contains a high percentage of a physiologically good protein, a considerable amount of energy-yielding material in the form of fat and carbohydrate, and a fairly liberal supply of the fat-soluble food accessory, as well as of the water-soluble growth determinant.

Abderhalden (1) found that rats, fed on soy beans, lived from 104 to 315 days. Two of them even increased in weight. Berczeller (3) also fed rats on raw whole soy bean, soy bean flour, and his patent soy bean flour "O." The rats were able to make a choice of any of these three foods. The results showed a duration of life of 7 to 55 days. The shortest life period corresponded to a high consumption of common soy bean flour, while a higher consumption of raw whole soy bean and of patent soy bean flour O was associated with an increase in the duration of life. The milling process evidently produces a significant change in the grain. Newburgh (24) found that four out of five rabbits after living on soy beans for 2 months showed no changes in the kidneys. But in a period from 4½ to 12 months they regularly acquired chronic renal lesions. These animals had a nitrogen metabolism about twice normal. The blood urea nitrogen showed a rise to 100 mg. per 100 cc. in 7 weeks, while the average value in the normal control period was 31 mg.

Nuzum, Osborne, and Sansum (25) placed a group of rabbits on a 38 per cent soy bean protein diet for a year, in order to study its relation to the production of hypertension. In addition to the soy beans, cod liver oil, tomato, and alfalfa were given. The soy bean group showed an elevation of blood pressure and changes in the blood and urine, while the

alkaline ash of the soy bean kept the urine alkaline. The carbon dioxide-combining power of the blood serum remained high. Albumin was not found regularly in the urine until almost a year after the protein feeding was commenced, although an increase in the non-protein nitrogen and urea nitrogen of the blood was noted in the 9th month.

In order to determine the biological value of the soy bean it was important to know the changes which a diet of soy beans produces in the chemical composition of the blood.

EXPERIMENTAL.

Seventeen normal rabbits were fed on a preliminary diet of boiled millet and raw cabbage, their blood taken from the marginal ear vein, and analyzed for glucose by Folin and Wu's method, urea nitrogen by the aeration method, cholesterol by Myers and Wardell's method, inorganic phosphorus by Benedict and Theis' method, and uric acid by Benedict's method. Later the animals were put on a diet consisting exclusively of yellow soy beans soaked in water for 15 hours, and the composition of the blood determined from time to time. Food was always present in the cages so that the animals ate more or less constantly. The results are presented in Tables I, II, III, and IV.

The chemical data show that in general there was an increase in blood urea, cholesterol, inorganic phosphorus, and uric acid, following raw soy bean ingestion. The glucose content was normal or high (minimum 133 mg. per 100 cc.).

Rabbits 5, 8, 9, and 11 died of pneumonia; Rabbits 3, 4, and 6 were killed; Rabbits 1 and 7 died; Rabbits 2, 10, 12, and 15 died of an unknown cause (nothing abnormal was found at autopsy); and Rabbits 13, 14, 16, and 17 were still alive at the end of the experiment. The bodies of the autopsied rabbits showed general deprivation of fat. In Rabbits 4 and 6 the gastric mucosa showed areas of desquamation, indicating an old healed inflammatory process. A pathological study of the kidneys of Rabbits 1 to 7 was done by Dr. J. R. Cash of the Department of Pathology, and his findings follow.

Autopsies upon these rabbits do not allow one to draw any definite conclusions as to the effect of a diet of soy beans upon the kidneys and other organs. In several of the animals there was almost total macroscopic disappearance of the body fat but in most cases the rabbits were in a very good state of nutrition.

TABLE I.

Cholesterol (Whole Blood) in Mg. per 100 Cc.

Rabbit No.....	1	2	3	4	5	6	7	Food.
Apr. 10	73	67	65	52	55	43	57	Millet and cabbage.
" 15	69	58	51	52	62	44	57	
Apr. 22	77	63	52	75	86	92	67	Raw soaked soy beans.
" 28	80	74	84	120	87	87	77	
May 6	77	Died	80	49	77	64	50	
" 13	71	May 4.	71	37	75	67	65	
" 18	71		55	80	80	63	52	
June 3	80		Killed	Killed	Died	Killed	80	
" 10	97		May 19.	May 19.	June 2.	May 19.	65	
" 24	63						75	
	Died July 5.						Died July 29.	

TABLE II.

Urea Nitrogen (Whole Blood) in Mg. per 100 Cc.

Rabbit No.....	1	2	3	4	5	6	7	Food.
Apr. 10	17.60	17.60	21.40	20.00	13.05	21.40	20.00	Millet and cabbage.
" 15	15.00	16.70	16.70	25.00	15.80	20.00	16.70	
Apr. 22	19.50	30.00	22.50	37.50	22.50	37.50	37.50	Raw soaked soy beans.
" 28	46.00	54.30	37.50	46.00	23.00	31.50	37.50	
May 6	50.00	Died	24.00	46.20	25.00	30.00	26.10	
" 13	40.00	May 4.	40.00	46.00	30.00	42.80	42.80	
" 18	46.00		43.00	54.50	26.00	37.50	50.00	
June 3	40.30		Killed	Killed	Died	Killed	46.00	
" 10	37.00		May 19.	May 19.	June 2.	May 19.	40.00	
" 24	37.50						46.20	
	Died						Died	
	July 5.						July 29.	

TABLE III.

Inorganic Phosphorus (Blood Serum) Mg. per 100 Cc.

Rabbit No.....	8	9	10	11	12	13	14	15	16	17	Food.
Sept. 10	7.12	7.12	7.12	6.25	4.35	5.10					Millet
" 16	7.12	6.90	5.11	5.53	4.25	4.76					and
" 22							4.35	4.35	4.57	4.00	cab-
" 24	6.23	5.88	5.50	5.88	4.76	5.00					bage.
Sept. 29							4.17	5.00	4.76	5.00	
Oct. 1	10.0	9.10	6.63	7.70	5.53	5.53					
" 6							4.55		5.00	4.35	
" 8	8.32	Died	6.23	4.17	5.88	5.88					Raw
" 13	Died	Oct. 4.	5.88	Died	5.00	4.65	5.88	5.00	5.26	5.00	soaked
" 20	Oct. 11.		6.06	Oct. 10.	5.26	5.00	5.00	6.66	5.00	5.40	soy
" 29			Died		Died	6.25	5.40	Died	4.60	4.76	beans.
			Oct. 24.		Oct. 26.			Oct. 26.			

TABLE IV.

Uric Acid (Blood Serum) Mg. per 100 Cc.

Rabbit No.....	8	9	10	11	12	13	Food.
Sept. 10	3.16	3.08	2.72	2.86	2.79	3.00	Millet and cabbage.
" 16	3.13	3.00	2.72	2.40	2.40	2.72	
" 24	2.85	3.00	2.72	2.72	2.50	2.60	
Oct. 1	3.53	3.00	3.17	3.17	3.42	3.75	Raw soaked soy beans.
" 8	4.00	Died	3.00	2.85	2.73	3.00	
" 13	Died	Oct. 4.	3.00	Died	3.00	2.67	
" 20	Oct. 11.		3.66	Oct. 10.	3.80	3.36	
" 29			Died Oct. 24.		Died Oct. 26.	3.07	

The kidneys as a rule were swollen and rather pale and in one case (Rabbit 1) were diffusely sprinkled with minute hemorrhages. There were no other macroscopical lesions noted. In no case was there any evidence of arteriosclerotic changes.

Microscopically the kidneys of Rabbit 1 showed many convoluted tubules filled with fresh red blood cells, older casts composed of blood, blood pigment, and desquamated epithelial cells. The epithelial lining remaining in such tubules showed marked infiltration with neutral fat. Here and there were small groups of convoluted tubules and loops of Henle where much fat was present in the epithelium, and a little coagulated albumin was seen in the lumina. The glomerular pores contained a few red blood cells. There was no evidence of the presence of an inflammatory process of any kind.

In Rabbit 7 the convoluted tubules were swollen but practically entirely intact. In a few of these loops and occasionally in a collecting tubule there were small calcified masses which probably represent epithelium which has sometime previously become necrotic. There was no fat or other acute change of any kind present.

In the remaining rabbits a few small areas of mononuclear infiltration were seen and a few of the glomeruli showed delicate adhesions to the capsule. Aside from these rather minor alterations the kidneys seemed normal in all respects.

DISCUSSION.

The yellow soy bean contains an average of 35 per cent of protein, 18 per cent of fat, 27 per cent of nitrogen-free extract, 5 per cent of fiber, 5 per cent of ash, and 10 per cent of moisture. Osborne and Mendel (26) found the proteins of the soy bean, unlike those of the other leguminous seeds thus far investigated, adequate for promoting normal growth. The soy bean is the only seed known to contain both water-soluble and fat-soluble vitamins. Horneman (14) has recently confirmed these findings.

In the present experiments the rabbits were, therefore, fed on a nearly complete food, especially when we take into consideration the fact that the soy beans were soaked for 15 hours, a sufficient time for the accumulation of the antiscorbutic factor.[1] The diet was deficient only in NaCl and Ca. A demonstration of the NaCl deficiency was given by five rabbits of the present series who on the death of Rabbit 9 were found to have torn open

[1] Berczeller's (3) findings in connection with the duration of life of animals fed on raw soy beans cannot be compared with these because the soy beans used here were thus soaked.

its back and bladder and to have consumed the urine completely, leaving the rest of the body untouched.

The normal and occasional high figures for blood sugar (the blood was taken from non-fasting animals) show that soy beans are not deficient in glucose-yielding substances.

Cholesterol.

Horvath and Chang (15) showed that there is an increase of the lipase content in the blood of rabbits fed on raw soy beans. Blood lipase hydrolyzes not only true fats but also other esters of the fatty acids, including lecithin. According to Bloor (6) the unsaturated fatty acids of blood plasma are found largely in combination with cholesterol. A lipase may, therefore, hydrolyze such cholesterol esters with the liberation of free cholesterol. The latter, if deposited in the walls of the blood vessels, might cause arteriosclerosis. This was one of the problems upon which it was hoped the present experiments would throw light.

In our rabbits the cholesterol content of the blood showed a general tendency to rise and reached in two animals twice the initial value.[2] Examination of the kidneys, however, showed no changes in the walls of the blood vessels which would lend support to any conclusion as to the occurrence of sclerosis.

The increase in cholesterol is probably due, at least in part, to the phytosterols known to be present in soy beans.

Some investigators have supposed that beriberi is an intoxication with cholesterol. Lawaczeck (cited by Kodama (17)) found that cholesterol was increased in the blood of pigeons fed on a diet deficient in vitamin B. In our experiments the rabbits received a food very rich in vitamin B, but, nevertheless, the cholesterol content of the blood was not reduced to a normal level. It seems, therefore, probable that vitamin B is not directly connected with the metabolism of cholesterol.

Urea.

The high figures for urea nitrogen which were obtained in some of our animals even after 1 week of the soy bean diet and after

[2] According to Baumann and Holly (2) the average cholesterol content of normal rabbit blood is 67 mg. per 100 cc.

2 weeks in six rabbits out of seven, can hardly be attributed to the high protein content of the soy bean, because in the experiments of Nuzum, Osborne, and Sansum (25) on rabbits placed on a diet containing 38 per cent soy bean protein, the urea nitrogen did not rise significantly above the average value during the control period (26 mg.) for 3 months and in a year reached a maximum of only 48 mg. On the other hand the microscopic examination gives no reason to believe the high urea content was due to pathological changes in the kidneys of Rabbits 3, 4, 5, and 6, fed on soy beans for 5 weeks. Newburgh (24) reports that in a similar experiment four out of five rabbits showed no changes in the kidneys at the end of 2 months, although the blood urea nitrogen increased to as much as 100 mg. in 7 weeks. We have, therefore, to seek in our case for another explanation. It seems possible that the increase of urea is associated with the high lecithin content of the soy bean, amounting to 1.64 per cent. Marie (22) found in rabbits that subcutaneous injections of lecithin and cholesterol were followed by a four- to sixfold rise in the concentration of blood urea. This may be the explanation of certain cases of high urea in the blood of Oriental people using soy beans as food.

However, another factor must be considered, namely soy bean urease. Carnot and Gerard (8) injected urease into dogs and obtained a disappearance of urea from the blood and a clinical picture of ammonia intoxication, confirmed by the determination of the blood ammonia. At first glance one might expect a drop in the blood urea of rabbits fed on raw soy beans, if the urease penetrates the blood; or, at least, an antagonistic action toward lecithin. Luck (20) found that urease exists in the animal body and recently showed its presence in the gastric mucosa of dogs. If we suppose the soy bean urease to exert its activity in the stomach, where the acidity is very favorable, we may expect penetration of the ammonia into the blood. It would reach the liver and other organs where ammonia is converted into urea. In case a part of the soy bean urease is absorbed by the capillaries of the intestinal tract (as was shown by Horvath and Chang (15) to be the case with soy bean lipase) and carried to the liver, it may aid in the conversion of ammonia into urea, since soy bean urease causes both the synthesis and the decomposition of urea (16, 21).

The presence in the soy bean of another enzyme, uricase (23),

which decomposes uric acid with the formation of urea, may also contribute to the increase in urea.[3]

Phosphates.

The inorganic phosphates increased in the majority of the cases. The soy bean contains as mentioned above 1.64 per cent of lecithin, and also a lipase capable of hydrolyzing lecithin. According to Koizumi (18) the introduction of lecithin into the blood results in a distinct increase in the inorganic phosphorus content. Rabbits 8, 9, 10, and 11 were young animals and while fed on millet and cabbage, the inorganic phosphorus content of their blood serum gradually decreased with age, as also occurs in children. Nevertheless, these rabbits gave a marked response to the soy bean food after a week, amounting in Rabbits 8 and 9 to 10.0 and 9.1 mg. per 100 cc. of blood serum, suggesting that the young organism is not able to regulate the inorganic phosphorus level as well as the adult. But in the course of time even the young animals showed a fairly good adjustment to the large amount of phosphorus ingested. Underhill and Bogert (27) showed that rabbits, in contrast to most other herbivora, excrete a considerable amount of phosphates through the kidneys. But they found that after successive subcutaneous injections of phosphate, less of the injected phosphorus was excreted in the urine than after the initial injection.

According to Bloor (5) lecithin probably takes an active part in fat metabolism as the first stage through which the fats pass in their utilization by the organism. The feeding experiments with lecithin performed by Hesse (13) on rabbits confirmed the finding that phosphatides possess a specific action in the deposition of fat. Koizumi (18) found that lecithin, injected intravenously, undergoes decomposition in the body. Its phosphorus in part appears in the blood as inorganic phosphate, and the fatty acids and glycerol unite to form fat. Diets high in fat may, therefore, deprive the body of its phosphorus, which is necessary for the conversion of fat into lecithin. The soy bean, however, is rich

[3] This observation must be taken into consideration in routine clinical laboratories as a possible source of error in the determination of urea nitrogen in blood by the aeration urease method.

in fat and at the same time rich in phosphorus, which is necessary for the metabolism of the former.

Hesse (13) found that vegetable lecithins (phytocithins) are not adequate for the deposition of fat. Berczeller's (3) patent soy bean flour O, containing an average of 20 per cent fat and 0.145 per cent lecithin does not produce obesity in human beings. The bodies of our rabbits fed on soy beans were found practically deprived of fat, but there may be some other explanation for this. In Rabbit 1 the epithelial cells of the tubules in the kdineys were filled with a fatty substance, although in this case the body was also deprived of fat. Newburgh (24) found that the chief deleterious effect of the soy bean diet was exerted upon the epithelium of the convoluted tubules.

Uric Acid.

According to Li Yu Ying (19), the soy bean is poor in nucleoalbuminoids yielding xanthine bases, but contains more paranucleoalbuminoids, which on decomposition do not yield purine bodies but only thymic acid. Some purine bodies are, of course, present in soy beans, but their amount does not seem to have been determined.

The increase in the uric acid content of the blood serum observed in these experiments may be due not only to the presence of purine bodies in the soy beans, but also to the large amount of fat present. Harding, Allin, Eagles, and Van Wyck (12) found that the blood uric acid is raised in human beings on high fat diets, sometimes to 10 mg. per 100 cc. These authors suggested that this increase may be partly accounted for by a decrease in blood volume with a consequent decrease in elimination. It would be simpler to suppose that the absorbed fat, in passing through the stage of lecithin before being utilized, takes the phosphorus from lipoid-like bodies and also from nucleic acid. The nucleins are a possible source of phosphoric acid and at the same time of protein, which may form non-protein nitrogenous bodies. In our results the figures for uric acid probably represent not only uric acid, but also some other closely related substances present in the blood (7). These substances may have originated partly from the soy beans or indirectly from the nucleins of the body, which the soy bean fat separated from their phosphoric acid. Harding, Allin, Eagles,

and Van Wyck (12) noted that on a high fat diet an increase sometimes occurs in the non-protein nitrogen, without any rise in the urea content of the blood. Recently Koizumi (18) has shown the formation of a monoaminomonophosphatide in the liver and also in the mucosa of the intestines. If the absorbed fat takes the phosphorus from such substances and the remaining nitrogenous compound is carried into the blood, an increase of the non-protein nitrogen would result. During muscular work it has been shown that there is a constant production of phosphoric and purine bodies. These substances may both arise in part from nuclein. On a diet high in fat, purine bodies may be liberated from nucleins in a form combined with a pentose. Such a purine pentose compound was found by Davis, Newton, and Benedict (10) in normal cow blood. This point of view is supported by the microscopic picture of lymphocytes taken from exudates of the peritoneal cavities of guinea pigs and rabbits, into which fats had been injected intraperitoneally by Bergel (4). These lymphocytes showed the ingested drop of fat to be closely embraced by the horseshoe nucleus. The fat to be metabolized is carried to the vicinity of nucleins, from which the phosphoric acid necessary for its conversion into lecithin is derived.

The presence of uricase in soy beans must also be taken into account, but the increase in uric acid shows that the combined effect of the purine bodies present in the soy bean and the high fat content is not counterbalanced by the uricase which was described by Němec (23) as "very active."

Rabbits 4 and 6, after being fed on raw soaked soy beans for 5 weeks were sacrificed and immediately autopsied. Desquamation of parts of the gastric mucosa was found and signs of a healed inflammatory process seen on gross examination. Horvath and Chang (15) report similar findings on a rabbit (V) fed on raw soaked soy beans for 9 days. Soy beans contain a very active proteolytic ferment. The mucosa of the stomach which is adapted to withstand the digestive effect of pepsin, may be unable to resist the action of a new proteolytic enzyme such as that of the soy bean. An inflammatory process may well result. In the course of time sufficient adaptation may occur and full resistance be established. This is suggested by the findings in Rabbits 4 and 6 in which after 5 weeks a healed process was present.

SUMMARY.

1. The blood of rabbits fed on raw soaked soy beans as a single food shows an increase in urea, cholesterol, inorganic phosphorus, and uric acid.

2. The possible sources, direct and indirect, of these increases are discussed.

3. Autopsies showed the body fat to be meager. No arteriosclerosis was found.

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LIPID* EXCRETION.

III. FURTHER STUDIES OF THE QUANTITATIVE RELATIONS OF THE FECAL LIPIDS.†

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The presence of fatty substances in the feces, under some conditions at least, has been known over a hundred years, a number of interesting observations on the subject having been recorded by Home (1) in 1813. In 1884 Müller (2), in the course of a general discussion of intestinal excretion, arrived at the conclusion that, inasmuch as fatty material is present in large amount in fasting feces and meconium, it, like other constituents, must represent some sort of an excretion. Numerous analyses of fecal lipids may be found in the literature; but practically all of them (with the exception of a few concerned with the sterols, to be discussed later) have been primarily concerned with various phases of the question of lipid absorption. Many of the authors have determined simply total, ether-extractable material excreted; others have investigated the relationships between the neutral fats, soaps, and sterols; no one appears to have made a detailed study of the fatty acids of the feces with the possibility in mind that valuable information with a bearing on the problems of lipid metabolism might thus be obtained. It seemed worth

* The term "lipid" is used in accordance with the recommendation of the International Congress of Pure and Applied Chemistry to include the fats and all substances ordinarily associated with them; *i.e.*, neutral fat (esters of fatty acids and glycerol), fatty acids, soaps, phospholipids, sterols, and esters of the sterols and fatty acids.

† The material presented in this paper is taken in greatest part from a thesis presented by the author to the faculty of the University of Rochester in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

while, therefore, to make such a detailed examination of fecal lipids, particularly of the fatty acids, in the hope of discovering the source and the purpose of the excretion. The possibility that unusual fatty acids might be found was especially attractive. The identification, for example, of fatty acids with an uneven number of carbon atoms, or with double bonds in positions where they do not ordinarily occur, or even the discovery of mixtures in which the solid and liquid acids were present in considerably different ratios from those in which they usually occur in the body, would have a very important bearing indeed upon problems of intermediary fat metabolism. From this standpoint the results so far have been negative. Though the possibility that unusual fatty acids are present has not been ruled out entirely, the data collected indicate that the fatty acids of the feces are such as may be found almost anywhere in the body and that they are present in about the same proportions as in the tissues. In a recent paper Eckstein (3), using the same methods, has obtained values for human stored fat very similar to those found for the fecal lipids.

In the previous papers of this series (4, 5) it was reported that, when moderate amounts of olive oil and coconut oil were fed, the lipids of the feces were to a large extent independent of the dietary lipids, although there was some similarity, evidenced by a comparatively high ratio of liquid to solid acids in the fecal lipids with an olive oil diet and the reverse with coconut oil. There was also a decided, though not large, increase in the total lipid excretion with diets containing lipids above that when the animals were receiving no lipids in their food. A considerably smaller excretion during fasting than with a lipid-free diet and a rather marked similarity between the lipids of the blood and feces were noted. From these results it appeared most probable that there is a basal excretion of lipid material, most nearly represented by that obtained with a lipid-free diet, and that this basal excretion is augmented by a small amount of lipids escaping absorption, or being absorbed and reexcreted, in the case of diets containing lipids. It seemed most desirable, therefore, in continuing the work, to make a more complete and careful study of the excretion with a lipid-free diet, and it is with such an investigation that the present paper is chiefly concerned.

EXPERIMENTAL.

General Procedure, Diet.—The technique of handling the dogs (used exclusively as subjects) and of collecting feces was the same as described in the preceding paper except for the use of alcohol to cover the excreted material during the collection period in the hope of reducing the oxidation of highly unsaturated acids.

The casein used in the diet was extracted with hot alcohol for 16 hours, either in the Clarke extractor (6) or a modification suggested by Dr. W. R. Bloor of this laboratory. The principal

FIG. 1. Continuous extraction apparatus. *A*, round bottom flask used as condenser; *B*, percolator; *C*, flask containing solvent; *D*, electric stove.

change from the original in the latter apparatus, a diagram of which is shown in Fig. 1, is the replacement of the cumbersome funnel (employed as container for the solid being extracted in the Clarke extractor) by a percolator, thus allowing the use of a much smaller flask as condenser. The material to be extracted is supported in the percolator (*B*), either in a cloth bag or fluted filter paper (a cloth bag is used with casein), and three or four glass tubes are placed in a vertical position around the bag to allow the alcohol vapor to pass up to the condenser (*A*). The tubes are especially important when casein is being extracted

since this substance swells considerably when wet with alcohol. A small funnel may be placed in the bottom of the percolator to aid in supporting the material and prevent its stopping the neck. Either a 500 or 1000 cc. flask, depending on the size of the percolator, is used as condenser.

The completeness of extraction of lipids was determined by digesting samples of the treated casein with 25 per cent NaOH on the steam bath for about 4 hours, acidifying with H_2SO_4, extracting with ether, distilling the ether, extracting the residue left by the ether with petroleum ether, and weighing the material thus extracted after distilling the petroleum ether and drying. Negligible amounts of lipids were found by this method, but, by accident, after most of the work to be reported had been completed, a sample was allowed to digest with alkali on the steam bath for a somewhat longer period of time (about 7 hours) and here a very appreciable amount of lipid substance was obtained amounting to 0.412 per cent. Further tests were carried out at once on casein which had been extracted 16 hours and was considered to be lipid-free and it was confirmed that there still remained about 0.5 per cent of lipids, which could only be removed after completely breaking down the protein molecule through long digestion with strong alkali. Analysis indicated that this substance consisted almost entirely of fatty acids, of which the average molecular weight was about 250 to 270. It was found that, if the casein were reextracted as before with alcohol for a period of several days, it was possible to remove completely all traces of lipids as shown by 24 hour digestion with 25 to 30 per cent NaOH and extraction with ether and petroleum ether as outlined above. Later work has shown that the same end may be accomplished by two or three much shorter periods of extraction, provided that the casein is removed from the apparatus, pulverized, and dried between them, and it is thus indicated that the failure to extract the lipids completely the first time is simply due to incomplete penetration of the alcohol to all parts of the mass of casein. If this be true, however, it is difficult to understand why it is necessary to break the protein down by long treatment with strong alkali in order to free the fatty substances so they may be extracted by ether. The whole matter presents an interesting problem which is to be made the subject of a later communication.

As far as the present paper goes we are principally concerned with the effect which these overlooked lipids may have had on the results of the experiments to be reported. In the first place one would hardly expect that any of such small amounts of lipids, amounting to an average of about 0.25 gm. per dog per day, would escape absorption; but in order to be sure that the lipids found in the feces with lipid-free diets, in which casein containing these small amounts of lipids was used, did not come from unabsorbed casein lipids, two of the dogs employed in these experiments were fed with the same diet used before, but with strictly lipid-free casein, for a period of 2 weeks and the feces were divided into weekly periods by charcoal demarcation and analyzed according to the methods described below. The results, the average values of which will be found in Table I (Lipid-free 5), were in general about the same as those obtained with casein which was not strictly lipid-free. Unfortunately there was a considerable fall in the total lipid excretion in the case of one of the dogs during the 2nd week, the amount excreted being somewhat less than that usually obtained. There was also a decrease in the proportional amount of solid acids in the 2nd week with both animals. It is thought, however, that these changes are due to normal variation in the animals and in view of the general similarity of the results to those obtained with casein, which was not strictly lipid-free, it seems unlikely that the small amounts of lipids present could have had any effect on the results and it will be assumed that the diet was to all intents and purposes strictly lipid-free.[1]

The diets were computed on the basis of 80 calories per kilo but it was found necessary to multiply the amounts by $\frac{9}{8}$ in the case of 5 to 7 kilo dogs and by $\frac{8.5}{8}$ in the case of 7 to 9 kilo dogs.

[1] In another series, which was continued for a period of 3 weeks and in which strictly lipid-free casein was used, three dogs continued to excrete lipids up to the end of the experiment without any appreciable diminution in the amount. The average total excretion was about 2 gm. per week and the composition was similar to that of the material excreted when the casein of the diet was not strictly lipid-free. This experiment was carried out as a control in work with bile fistula animals and will be published with the data from that investigation.

TABLE I.

Average Composition of Fecal Lipids.

Experiment.	No. of dogs used.	Weight of dogs.	Total lipids.	Unsaponifiable.		Non-volatile fatty acids.			Solid fatty acids.				Liquid fatty acids.			Ratio of liquid to solid fatty acids.
				Weight.	In total lipids.	Weight.	In total lipids.	Melting point.	Weight.	In total lipids.	In non-volatile fatty acids.	Melting point.	Weight.	In total lipids.	In non-volatile fatty acids.	
			A	B	100B/A	C	100C/A		D	100D/A	100D/C		E	100E/A	100E/C	E/D
		kg.	*gm.*	*gm.*	*per cent*	*gm.*	*per cent*	*°C.*	*gm.*	*per cent*	*per cent*	*°C.*	*gm.*	*per cent*	*per cent*	
Low		5.4	3.636	1.464	36.9	2.046	56.5	37	0.408	10.7	17.2	49	1.476	38.4	61.0	3.20
Olive oil 1.	3	8.8	3.840	1.498	39.1	2.302	59.8	38	0.438	11.4	19.2	50	1.516	39.5	66.3	3.40
High		13.8	4.074	1.526	42.0	2.566	63.0	40	0.465	12.8	22.7	50	1.561	40.5	72.0	3.70
Low		5.4	2.181	0.902	38.6	1.207	47.5	27	0.181	7.0	13.1	47	0.826	32.3	65.9	3.22
Olive oil 2	6	8.8	3.309	1.437	43.4	1.693	51.2	28	0.300	9.0	17.6	50.2	1.215	36.6	71.4	4.23
High		12.9	4.298	1.851	46.6	2.271	55.2	29	0.464	10.8	21.2	52	1.602	43.6	81.0	6.20
Low		5.1	0.552	0.242	43.8	0.284	35.2	36	0.025	4.5	8.3	48	0.241	29.8	70.0	2.60
Fasting	5	9.2	0.948	0.503	52.9	0.436	46.2	36.5	0.096	9.1	20.0	48.5	0.350	38.5	82.7	5.26
High		13.2	1.608	0.834	65.0	0.762	54.7	37	0.213	13.3	28.0	49	0.545	54.0	98.5	11.90
Low		4.8	1.955	0.643	24.9	1.290	66.0	34	0.567	23.6	32.4	45	0.683	32.8	46.7	0.88
Coconut oil	6	8.1	2.859	0.860	30.3	2.019	70.3	37	0.868	30.5	43.5	47	1.037	36.1	51.4	1.21
High		12.1	4.217	1.197	34.6	2.937	76.1	38	1.273	37.2	53.0	48	1.534	39.5	54.4	1.67
Low		5.9	1.515	0.582	31.4	0.851	56.0	34	0.249	13.8	23.0	50	0.555	36.6	62.5	2.19
Lipid-free 1	4	9.5	2.174	0.793	36.9	1.263	56.5	37	0.340	15.9	27.4	50.5	0.829	37.8	65.2	2.40
High		13.6	2.852	1.012	40.5	1.706	59.7	40	0.406	17.1	29.2	51	1.147	40.1	67.1	2.92

Low.....		5.3	1.329	0.565	36.8	0.674	47.0	33	0.225	14.1	25.7	49	0.422	28.6	57.1	1.75
Lipid-free 2 A	5	7.5	2.077	0.853	41.3	1.088	51.9	36	0.325	15.7	30.1	51	0.672	32.4	61.7	2.08
High.....		10.9	3.177	1.170	45.0	1.850	58.2	42	0.501	17.2	34.7	52	1.112	36.7	67.8	2.44
Low.....		5.3	1.807	0.708	39.2	0.857	38.2	*	0.202	9.0	20.8	48	0.536	23.8	59.3	2.34
Lipid-free 2 B.....	4	6.7	1.971	1.022	50.9	0.964	49.7	*	0.220	11.3	23.1	50	0.525	29.9	60.6	2.64
High.....		9.0	2.243	1.444	64.3	1.096	60.6	*	0.228	12.6	26.0	51	0.654	36.2	62.6	2.86
Low.....		5.5	1.176	0.597	36.6	0.525	44.6	*	0.140	9.6	19.0	47	0.369	25.2	46.2	2.16
Lipid-free 2 C.....	4	7.9	1.599	0.709	45.7	0.795	48.8	*	0.181	11.5	23.9	49	0.489	31.2	64.4	2.79
High.....		10.8	2.193	0.801	51.8	1.198	54.5	*	0.233	13.8	30.5	50	0.595	38.6	75.6	4.00
Low.....		7.0	1.280	0.377	28.2	0.815	58.0	32	0.242	18.9	29.7	46	0.505	32.3	55.6	1.42
Lipid-free 3 A.....	5	9.5	1.974	0.596	30.0	1.255	63.6	35	0.348	20.5	32.6	47	0.624	37.5	59.3	1.85
High.....		12.9	3.152	0.973	33.0	2.044	66.5	39	0.473	22.8	39.4	50	0.669	40.3	62.0	2.09
Low.....		6.6	1.431	0.497	29.6	0.867	55.5	38	0.284	19.2	31.1	46	0.493	25.6	41.5	1.33
Lipid-free 3 B.....	5	9.1	1.872	0.598	32.3	1.127	60.0	39	0.378	20.3	34.0	47.4	0.594	32.0	53.4	1.57
High.....		12.5	2.411	0.716	35.3	1.505	62.5	41.5	0.468	22.8	38.2	49	0.842	35.3	59.0	1.80
Low.....		6.2	1.033	0.405	28.4	0.648	57.0	36	0.218	21.1	33.6	49	0.333	27.2	47.4	1.20
Lipid-free 3 C.....	4	9.1	1.594	0.555	35.3	1.029	64.1	37	0.372	23.0	36.1	50	0.513	32.2	50.1	1.40
High.....		12.0	2.003	0.670	40.4	1.369	68.3	37	0.483	24.3	39.8	50	0.446	37.0	54.0	1.53
Low.....		7.2	1.316	0.413	31.4	0.871	59.1	36	0.304	21.2	34.9	47.5	0.460	29.8	48.5	1.28
Lipid-free 3 D.....	4	8.8	1.940	0.676	34.1	1.185	61.6	37.5	0.439	22.6	36.6	48.6	0.615	32.1	52.1	1.43
High.....		11.1	2.622	0.978	37.2	1.553	66.4	38.5	0.594	23.3	38.2	50	0.817	35.0	54.5	1.54

TABLE I—*Concluded.*

Experiment.	No. of dogs used.	Weight of dogs.	Total lipids.	Unsaponifiable.		Non-volatile fatty acids.			Solid fatty acids.				Liquid fatty acids.			Ratio of liquid to solid fatty acids.
				Weight.	In total lipids.	Weight.	In total lipids.	Melting point.	Weight.	In total lipids.	In non-volatile fatty acids.	Melting point.	Weight.	In total lipids.	In non-volatile fatty acids.	
			A	B	$\frac{100B}{A}$	C	$\frac{100C}{A}$		D	$\frac{100D}{A}$	$\frac{100D}{C}$		E	$\frac{100E}{A}$	$\frac{100E}{C}$	$\frac{E}{D}$
		kg.	*gm.*	*gm.*	*per cent*	*gm.*	*per cent*	*°C.*	*gm.*	*per cent*	*per cent*	*°C.*	*gm.*	*per cent*	*per cent*	
Low		5.4	0.730	0.202	32.6	0.414	56.6	34	0.148	15.7	26.1	48	0.264	36.2	63.0	1.78
Lipid-free 3 E	3	6.4	1.332	0.456	35.3	0.810	60.1	36	0.245	18.8	31.4	48.2	0.516	38.3	63.7	2.06
High		6.9	1.724	0.561	40.0	1.032	63.8	38	0.318	20.6	35.8	49	0.662	40.3	64.0	2.45
Low		7.6	0.588	0.244	35.8	0.386	50.0	36.5	0.078	10.8	18.8	48	0.206	26.2	52.0	1.61
Lipid-free 4	5	10.2	1.410	0.587	42.7	0.787	57.6	37.4	0.206	13.9	24.3	49.5	0.426	31.4	54.4	2.33
High		13.1	2.600	0.931	50.0	1.392	65.5	38.5	0.448	17.2	32.2	51	0.724	35.5	57.6	3.00
Low		7.9	0.974	0.323	27.8	0.626	52.4	35	0.134	11.7	21.4	45.5	0.294	23.6	44.7	1.19
Lipid-free 5	2†	8.0	1.981	0.662	33.2	1.124	58.0	37.8	0.347	16.6	28.9	47.6	0.521	27.0	46.6	1.72
High		8.4	2.781	1.027	36.8	1.469	64.5	39	0.552	21.3	37.6	49.5	0.656	30.2	47.8	2.20

* Material too dark for melting points to be seen.

† Four weekly determinations.

The composition of the diets on the basis of 80 calories per kilo, expressed in gm. per kilo per day, was as follows:

	Lipid-free 2.	Lipid-free 3.	Lipid-free 4.	Lipid-free 5.
Casein	6.4	6.4	6.4	6.4
Sugar	13.6	13.6	13.6	13.6
Bone ash	0.4	0.4	0.4	0.4
Meat extract	0.5		0.5	0.5
Lettuce		3.0		
Water	1.75	2.0	1.75	1.75

In order to eliminate any possible influence of stagnation or incomplete demarcation of the feces it seemed desirable to carry out experiments of longer duration than the single week used heretofore and two such series have been conducted with lipid-free diets, one 4 weeks and the other 5 weeks in length. The feces were collected in weekly periods, charcoal being used for demarcation. The first of these series (Lipid-free 2) was marred by the abnormal condition of three of the dogs, one showing pathological symptoms during the 3rd week and the others at the beginning of the 4th week of the series. They appeared to be suffering from distemper, of which there was an epidemic in the kennels at the time, accentuated perhaps by dietary deficiency. It had been intended at the start to continue the experiment for 2 weeks only and was, therefore, thought unnecessary to add accessory factors to the diet; but as the animals appeared to be normal and were eating well at the end of that time, it was decided to carry it on for a longer period.

In the second series (Lipid-free 3), with the exceptions noted below, the animals were normal throughout, no pathological or unusual symptoms, aside from loss of weight, having been observed. One dog, not being accustomed to experimental diets, ate rather poorly from the beginning and refused to eat at all after the 4th week. No data were obtained from this dog during the 5th week. Another dog was pregnant and aborted during the 4th week. Though the values obtained with this animal were for the most part in close agreement with the averages from the others, they are not included in the averages because of the possible effect of the abnormality of the dog. Lettuce was included

in the diet because it offered the advantage of supplying vitamins with a minimum of fat. It has been found in this laboratory to maintain dogs in health for a period of at least 4 weeks.

Analytical Methods.—The analytical procedure was the same as that described in the preceding paper with a few minor changes, one of which had as its object the more complete recovery of the volatile acids and consisted in distilling the filtrate from the acidified digestion mixture of the feces at once before extraction. After removal of volatile acids the solids from the filtration and the residual liquors from distillation were separately extracted with ether; the ether solutions were combined, washed with water, concentrated, and finally dried on the water bath. The residue was extracted with petroleum ether to obtain the non-volatile lipids.

Two slight modifications were also made in the separation of solid from liquid acids. Ether was substituted for petroleum ether as extracting agent because it seemed to show less tendency to cause emulsions, and HNO_3 (2:50) was substituted for HCl in decomposing the lead soaps of the liquid acids because of the trouble caused by insoluble $PbCl_2$. In order to determine the possible effect of HNO_3 in oxidizing unsaturated acids, the following experiments were performed. Three equal portions of linseed oil were carried through the procedure used in the separation of the fecal lipids, all being treated in the same manner except for the use of HNO_3 (2:50), HCl (2:50), and acetic acid (2:50) respectively in setting free the liquid acids from their lead soaps. The ether solutions of the fatty acids were allowed to stand in contact with the acid solutions for 24 hours—a longer period than was ever used in the analysis of the fecal lipids. The iodine numbers of the acids thus obtained were as follows:

Acid used.	Iodine No.
HNO_3	126
HCl	116
CH_3COOH	118

These results indicate that HNO_3 in the concentration used has no more oxidizing effect than the other acids. The low iodine numbers show incidentally that there is much opportunity for oxidation in the method and that, therefore, in all probability

only a portion of the highly unsaturated acids of the feces are recovered.

In another experiment three equal portions of linseed oil were dissolved in equal volumes of ether without preliminary treatment and shaken in separatory funnels with equal volumes of the acid solutions used before. The mixtures were allowed to stand 4 days, after which the iodine numbers of the three samples were determined. Great care was exercised in giving all the samples as nearly the same treatment as possible throughout. The following iodine numbers were obtained:

Acid used.	Iodine No.
HNO_3	168
HCl	167
CH_3COOH	178

These experiments taken together seem to demonstrate that HNO_3 in the concentration employed has no appreciable oxidizing influence and, in fact, no effect was seen in the recovery of highly unsaturated acids.

Computation of Data and Explanation of Table I.—It would perhaps seem most reasonable to compute the results in terms of excretion per unit body weight; but the variations between animals and in the same animal during different weeks, so far as total excretion goes, have been found to be so great in all of this work that there seemed to be no point in the use of such a method. The data have been computed, therefore, in terms of total weekly excretion per animal. Inasmuch as the same dogs were used in most of the experiments, this method of treatment gives quite comparable results.

Because of lack of space it is not possible to include the values obtained with individual animals. The results of each weekly experiment have been averaged and the averages together with the maximum and minimum values are given in Table I. The letters are used in connection with the long series to designate the week of the experiment; *e.g.*, Lipid-free 2 B includes the data obtained in the 2nd week of the series called Lipid-free 2.

Because of the failure to show any relationship between the volatile fatty acids and non-volatile lipids, the loss of volatile fatty acids during acidification of the fecal digestion mixtures,

and the necessity of analyzing the volatile fraction for each animal separately when it is to be added in with the non-volatile lipids, it has seemed more and more desirable as the work has progressed to confine the attention to the non-volatile lipids. No attempt has been made, therefore, to include values for the volatile acids in the "total lipid" fraction as was done in the preceding paper (see discussion of volatile acids below) and "total lipids" include only total non-volatile lipids. The data from some of the experiments reported in the preceding paper have been recomputed with the omission of volatile acids. The results thus obtained are much more concordant and the average values are included in Table I for comparison.

In Table I are also included the results from an experiment with a diet containing olive oil (Olive oil 2) carried out in the same manner as the one (Olive oil 1) described in the preceding paper, in order to confirm if possible the results of that work. In general the data are in complete agreement with those obtained in the earlier experiment.

Alcohol Extraction Method. Lipid-Free 4.—Continuing the idea of making a detailed examination of the basal excretion with lipid-free diets, an experiment was carried out in which the feces excreted by dogs during a week on such a diet were extracted in the modified Clarke apparatus described above with hot alcohol for 3 hours without preliminary saponification, after which they were removed, pulverized, and extracted 34 hours more. The method of analysis consisted briefly in separating the neutral and fatty acid portions by extraction from alkaline and acid solutions respectively with petroleum ether, saponification of the neutral fraction with alcoholic KOH, separation of the saponified neutral fraction into unsaponifiable and fatty acid portions by the usual technique, distillation of volatile fatty acids from the free fatty acid fraction, and separation of both the free fatty acids and the acids from the neutral portion into solid and liquid fractions.

The average values of the data, arranged to show the relationships brought out by the method of analysis, will be found in Table II. The data have also been computed for comparison with the results of the other experiments and the average values will be found in Table I.

TABLE II.

Lipid-Free 4 (Alcohol Extraction Method).

	Average.	Low.	High.
Weight of total lipids.....A	1.112	0.588	1.425
" " neutral fraction.....B	0.664	0.304	1.025
Percentage of neutral fraction in total lipids.....$\frac{100B}{A}$	58.1	49.9	72.0
Weight of unsaponifiable fraction.....C	0.501	0.244	0.711
Percentage of unsaponifiable fraction in neutral fraction.....$\frac{100C}{B}$	77.1	69.5	83.0
Weight of non-volatile fatty acids in neutral fraction.....D	0.194	0.111	0.321
Percentage of non-volatile fatty acids in neutral fraction.....$\frac{100D}{B}$	29.8	23.3	36.6
Weight of solid fatty acids in neutral fraction.....E	0.051	0.021	0.096
Melting point of solid fatty acids in neutral fraction.....	48.4	48	50
Weight of liquid fatty acids in neutral fraction.....F	0.101	0.056	0.157
Ratio of liquid to solid fatty acids in neutral fraction.....$\frac{F}{E}$	2.17	1.64	2.66
Weight of free fatty acids.....G	0.442	0.275	0.606
Percentage of free fatty acids in total lipids.....$\frac{100G}{A}$	41.1	27.2	50.0
Weight of solid fatty acids in free fatty acid fraction.....H	0.094	0.057	0.163
Melting point of solid fatty acids in free acid fraction.....	50.2	47.8	52.5
Weight of liquid fatty acids in free acid fraction.....I	0.251	0.150	0.355
Ratio of liquid to solid fatty acids in free fatty acid fraction.....$\frac{I}{H}$	2.78	2.18	3.32

Volatile Acids.—In previous work analysis of the volatile fatty acids by the method of Dyer (7) indicated that butyric was the principal one present and the weight of this fraction was computed on the assumption that it was made up entirely of butyric,

so that it might be added with the non-volatile lipids to give a value for total lipids. It was felt that, if such an addition were to be made, the volatile acids should be more accurately analyzed than was possible with the Dyer method, which does not pretend to give quantitative data when more than one acid is present, and the relatively accurate but tedious modification of the Duclaux method, proposed by Gillespie and Walters (8), was adopted. The samples for analysis were obtained by concentrating the volatile acid distillates after titration in alkaline solution, acidifying with H_2SO_4, and distilling almost to dryness. The principal difficulty in the method, aside from the laborious computations, is the necessity of discovering what acids are present by a cut and try procedure, it being fairly certain that the correct ones have been chosen when consistently positive results are obtained. Brieger (9) studied the volatile acids of the feces by means of their silver and guanidine salts and concluded that they contained 75 per cent acetic. He also demonstrated the presence of butyric and, as he thought, valerianic; but his evidence can be just as well interpreted as showing caproic. Edelstein and Csonka (10), using vacuum steam distillation to separate the volatile acids and fractionation of the silver salts to determine them quantitatively, found that in the feces of normal and dyspeptic infants they consisted in largest part of acetic with small amounts of butyric, caprylic, and formic present. The experiments with the Dyer method indicated the presence of butryic and small amounts of caproic. On the basis of the foregoing evidence it seemed reasonable to compute the results assuming that the volatile acids consisted of acetic, butyric, and caproic, and that this guess was correct is strongly indicated by the uniformly positive results obtained. In fourteen determinations upon feces from dogs on a lipid-free diet the following average values were obtained.

	Acetic.	Butyric.	Caproic.
	per cent	*per cent*	*per cent*
Low	44.30	9.89	3.64
Average	65.35	23.33	11.31
High	77.81	35.74	25.20

Though there is considerable variation in the results, they show perhaps as much uniformity as can be expected with an indirect method involving complex computations such as this. They are in general agreement with the work mentioned before and with the results recently reported by Cecchini (11). In studying fecal volatile acids in connection with their bearing on intestinal putrefaction he used the Duclaux method but did not apply any arithmetical treatment such as that of Gillespie and Walters. He simply placed the curve of the unknown showing speed of distillation between the two nearest curves of pure acids and assumed only these two acids to be present in inverse ratio to the distances from the curve of the unknown to the curves of the pure acids. In most cases he found these to be acetic and

TABLE III.

Mixture of acids from:	Palmitic.	Stearic.
	per cent	*per cent*
Lipid-free 3 A	49.6	52.5
" " 3 B	49.5	47.6
" " 3 C	41.5	62.5
" " 3 D	44.0	40.2
" " 3 E	37.2	53.0
" " 4 (free acids)	48.4	63.2
" " 4 (combined acids)	41.5	45.5
Average	44.5	52.1

butyric, the unknown curve lying about half way between the curves of these acids; but he could have explained his results as easily and probably more accurately by assuming that some caproic and less butyric was present.

Solid Acids.—The saturated fatty acids from some of the experiments were analyzed by the melting point method of Twitchell (12), using pure lauric, myristic, palmitic, and stearic acids manufactured by the Eastman Kodak Company for reference. No indication of the presence of lauric or myristic acids was found in any of the samples analyzed, the lowering of the melting points of these acids being a little greater in most cases with the fatty acids from the feces than with the same amount of pure palmitic. The results with palmitic and stearic are given in Table III.

The results are not as uniform as might be desired and in two cases total over 100 per cent, but this may be partially explained by the difficulty which was encountered in taking comparable melting points. The variation in reading was probably 0.2–0.3°, equivalent to an error of 5 to 10 per cent. It seems fairly certain that the solid fatty acids of the feces consist almost, if not entirely, of palmitic and stearic acids with the stearic present in slightly larger amount.

Liquid Acids.—The combined liquid acids from some of these experiments were separated by the barium soap-benzene method of Farnsteiner (13) to remove small amounts of solid acids which might have escaped removal before. The method was said by its author to separate oleic from more highly unsaturated acids; but according to Lewkowitsch (14) the separation is not nearly complete when an excess of oleic is present. The portion soluble in benzene, containing part of the oleic and more highly unsaturated acids, was brominated and separated into ether-soluble and ether-insoluble portions, which were analyzed for bromine, and in some cases carbon, by combustion in the Parr bomb. Where carbon was determined a weighed amount of benzoic acid was used as accelerator and the CO_2 formed was measured in the Parr (15) total carbon apparatus. Concentrated HNO_3 was used to generate the CO_2 instead of the HCl ordinarily employed in order that by simply adding ferric alum indicator bromine might be determined by the regular Volhard titration in the same sample. To check the apparatus and the method a sample of benzoic acid resublimed in the laboratory and another obtained from the Eastman Kodak Company were analyzed and gave the following results:

	Resublimed.	Eastman.	Theory.
Carbon, *per cent*	68.2	68.4	68.8

p-Bromoacetanilide also from the Eastman Kodak Company gave the following results:

	Found.	Theory.
Carbon, *per cent*	44.5	44.7
Bromine, " "	37.21	37.28

Melting points of the ether-insoluble bromides were determined in some cases, the figures given indicating the point at which the material blackened and fused together. It was found in qualitative tests that the ether-insoluble substance was also practically insoluble in chloroform and benzene.

The data are collected in Table IV together with some results from previous work and the composition and melting points of some common bromo acids are included for comparison. The evident lack of uniformity is probably to have been expected when it is considered that the syrupy nature of the ether-soluble and the small amount and insolubility of the ether-insoluble bromides precluded any attempt at purification. Moreover there was strong possibility of incomplete combustion of ether-soluble bromides due to the difficulty of mixing the syrupy material with the peroxide and accelerator in the bomb and also the probability of error in the analysis of the ether-insoluble bromides was great because of the indirect method necessitated by the small amounts available.

Though most of them are low, the bromine analyses appear to indicate that the ether-soluble fraction is made up mainly of dibromostearic acid and the syrupy nature of the substance leads to the same conclusion. On the other hand the carbon analyses seem to point to the presence of dibromopalmitic although the variations are so great that conclusions are difficult to draw. Though there is no evidence to show it, the possible presence of tetrabromo acids is by no means excluded.

In the previous paper it was stated that the ether-insoluble portion appeared from the analysis to consist of the hexabromide of an acid containing 18 carbon atoms. Its insolubility in chloroform and benzene and its high charring point force one, however, to the view that it is an octabromide and very probably the octabromide of arachidic acid. It is not difficult to correlate the other data with the assumption that this acid is present. There is not a great deal of difference, in comparison with the accuracy of the analysis, in the percentages of bromine and carbon in hexabromostearic and octabromoarachidic acids and, moreover, several of the bromine values are high for hexabromostearic while, due to the small samples used and the probable presence of impurities, we would expect low results. The carbon analyses prob-

TABLE IV.

Analysis of Liquid Acids.

Experiment.	Amount analyzed.	Fraction from insoluble barium soaps.		Fraction from soluble barium soaps.	Ether-soluble bromo acids.			Ether-insoluble bromo acids.			
		Weight.	Iodine No.		Weight.	Bromine content.	Carbon content.	Weight.	Charring point.	Bromine content.	Carbon content.
	gm.	*gm.*		*gm.*	*gm.*	*per cent*	*per cent*	*gm.*	*°C.*	*per cent*	*per cent*
Olive oil 1	5.167	1.257	62.5	3.324	4.287	36.02		0.1756		63.59	
Coconut oil	6.223	2.109	39.8	2.933	4.286	32.70		0.1762		62.74	
Lipid-free 1	2.564	0.311	46.5	1.322	1.807	34.46		0.1506		64.94	
Olive oil 2	6.875	1.977	62.5	4.633	6.617	28.75		0.0986	217	61.60	
Lipid-free 2 A	3.358	0.536	50.8	1.724	2.493	33.75	45.0	0.1418	214	61.60	28.8
" " 2 B	1.792	0.648	49.0	1.125	1.789			0.0633			
" " 2 C	1.558	0.857	56.4	0.671	1.043	35.40	43.3	0.0461		64.60	23.6
" " 2 D	1.003	0.269	78.6	0.591	0.920			0.0579			
" " 3 A	4.533	1.946	47.0	2.488	3.285	28.40	50.6	0.0393	215		
" " 3 B	3.192	0.758	46.5	2.221	3.188	36.10	46.7	0.1911	210	62.20	30.0
" " 3 C	1.777	0.882	66.6	0.866	1.379	35.0	42.0	0.0208			
" " 3 D	2.371	1.736	80.4	0.309	0.601	35.9	37.6				
" " 3 E	1.260			1.119	1.550	27.0	44.2	0.0265			
" " 4 (free acids)	1.775	0.476	68.0	1.005	1.523			0.0817	215		
" " 4 (combined acids)	1.035	0.243	71.0	0.712	1.008						

Composition of various bromo acids.

Acid.	Bromine.	Carbon.	Melting point.	Acid.	Bromine.	Carbon.	Melting point.
	per cent	*per cent*	°C.		*per cent*	*per cent*	°C.
Dibromostearic.....	36.18	49.00	Oil.	Hexabromostearic.....	63.32	28.47	180–181
Dibromopalmitic.....	38.66	46.50	29	Hexabromopalmitic.....	65.75	26.30	
Tetrabromostearic.....	53.33	36.00	113–114	Octabromostearic.....	69.80	23.60	Chars 200
Tetrabromopalmitic.....	55.94	33.60		Octabromoarachidic.....	67.87	25.41	" 220

ably do not signify a great deal for reasons mentioned except to show the general order of the carbon content, and the best of these, run on a fairly large sample of the combined bromo acids of three experiments, showed a result much too low for hexabromostearic but not far from that of octabromoarachidic.

Taking these data as a whole they seem to indicate that the liquid acid fraction is made up very largely of an acid or acids with one double bond—probably oleic—together with a small amount of highly unsaturated portion which consists almost entirely or arachidonic acid.

DISCUSSION.

One of the principal purposes of this investigation was that of determining the average composition of the lipids excreted with a lipid-free diet. Though there is a marked uniformity in composition exhibited in the case of the different animals in Lipid-free Series 2 and 3, a distinct difference between the series themselves is noticeable, the ratios of unsaponifiable matter to fatty acids and of liquid to solid acids being higher in Series 2 than in Series 3. In searching for a reason for the discrepancy one thinks of the pathological condition of some of the animals near the end of Series 2; but it is not likely that it could have affected the results of the first 2 weeks when the dogs were all normal. It seems more likely that the difference is due to the previous diet and condition of the dogs, for, though the normal laboratory diet, which preceded both of the series, is quite constant in composition, they were in considerably better condition at the start, as evidenced by comparative weights of dogs used in both series, in Series 3 than in Series 2. If this be true, however, it is difficult to see why the composition of the lipids excreted in Series 3 should not have approached that of Series 2 as the experiment went on and the animals lost weight. There appear to be other factors involved and it is deemed best, for the present at least, to strike a rough average between the two series, such as is shown by the results of Lipid-free 1, and consider it as the composition of the normal, basal lipid excretion.

The data obtained in these experiments present additional evidence in favor of the old view of Müller that a large proportion at least of the fecal lipids come from some other source than

unabsorbed food. The continued excretion of these substances over long periods of time by animals receiving no lipids in their food leaves little doubt concerning this point. When lipids are present in the food, they seem, however, to have an effect on the fecal lipids as shown by a larger total excretion and an increase in the proportion of liquid acids of the feces with an olive oil and in the solid acids with a coconut oil diet. The increase in unsaponifiable excretion with an olive oil diet is also traceable in all probability to the unsaponifiable matter of the olive oil. Such results suggest at once that we are dealing here with a basal excretion plus a certain amount of diet lipids which either have escaped absorption or have been absorbed and reexcreted as the result of a plethora such as might be expected if the fecal lipids represent some sort of leakage from the blood. In either case we would expect the lipids in excess of the basal excretion to be similar in composition to those of the diet and, in fact, when the values for the basal excretion, which we have assumed to be represented by those from Lipid-free 1, are subtracted from the corresponding values obtained in the olive oil experiments, the solid fraction cancels out leaving unsaponifiable and liquid acids in about equal proportions. It seems reasonable to assume that a large proportion of the unsaponifiable material has escaped absorption or, more probably, has been absorbed and excreted again, while a much smaller proportion of the liquid acids has suffered the same fate. When the same procedure is applied to the coconut oil experiment, there is found a small excess of unsaponifiable over the basal, a decided excess of solid acids, and also a smaller but definite excess of liquid acids. This last is difficult to understand on the assumption of a basal excretion plus some diet lipids unless we incline to the second view expressed above (that the increased excretion with lipids in the diet is due to a plethora) and explain it as being brought about by partial desaturation of the solid acids of the coconut oil.

There are several possible sources of the fecal lipids. The finding of lipids in the intestinal mucosa by Schmidt (16) suggests that they may come from desquamation of intestinal epithelial cells; but it may be that the lipids he found were simply in transit into the gut. The finding of large amounts of lipids in isolated intestinal loops where there could have been relatively little

desquamation of cells indicates that this is not the source. It is hoped that some light on this question may be obtained by experiments, which are being planned, involving the examination of the mucosa of the intestine for lipids by the methods used in this investigation. It is realized, however, that even if lipids are found, of the same composition as those of the feces, it will be difficult to say whether they formed a constituent of the cells or were merely on their way from the blood into the lumen of the intestine.

Another possible source is the intestinal bacteria, concerning the lipid content of which there is an unfortunately small amount of information available. Nencki and Schaffer (17) found a lipid content of dried putrefactive bacteria of 6.04 per cent while Kappes (quoted from Kendall (18)) found 4.8 per cent in *Bacillus prodigiosus*. Nicolle and Alilaire (19) found somewhat higher percentages using acetone and chloroform as solvents for extraction in a large variety of bacteria; but the recent work of Larson and Larson (20), who found even larger amounts of acetone extract, of which, however, only a small portion in most cases was soluble in petroleum ether, indicates that these results were in error. The last named authors computed as lipids the sum of the petroleum ether and ethyl ether extracts and found the percentage amounts on a dry basis to lie between 3.06 and 6.80 with one exception where a comparatively very small sample was used. These significant results were obtained with *Staphylococcus albus*, *Bacillus mucosus*, *Bacillus coli communis*, and *Bacillus coli communior*, all of which are normal inhabitants of the gut. From what evidence there is, it appears that the average lipid content of bacteria native to the intestine lies between 4 and 5 per cent. Kendall estimates that an adult human excretes about 5 gm. of dried bacteria per day, from which it hardly seems probable that the small dogs used in these experiments would excrete more than 1 gm. per day equivalent to 0.3 to 0.35 gm. of lipids per week at the maximum.

Further evidence that bacterial lipids form at most a small part of fecal lipids is presented by the fact that the sterile meconium contains much lipid material and by the finding of lipids in large amounts in isolated intestinal loops where bacterial life must have been reduced to a minimum. Kobert and Koch (21) ob-

tained lipids from a loop of the large intestine after they had sterilized it with antiseptic substances.

It seems to be the general opinion of those who have studied the fecal sterols that they reach the intestine either through the food or the bile. Gardner and coworkers (22), who have done a great deal of work with sterol balance, determining these substances in the food and the feces, were long of the opinion that cholesterol was not synthesized in the body; but they have recently reversed their opinion because of the finding in many cases of greater amounts of cholesterol in the feces than in the food. They recognized early in their work that a much larger amount of cholesterol was poured into the intestine through the bile than was excreted through the feces and they concluded that the cholesterol of the bile came from breakdown of red blood cells in the liver and was reabsorbed for use again elsewhere in the body, thereby forming a sort of closed circuit, while the sterols of the feces came entirely from the food sterols, which might or might not have been absorbed and reexcreted. They now believe that some of the bile cholesterol escapes reabsorption, perhaps as difficultly hydrolyzable esters, and that more is excreted than is taken in the food. The results of the present investigation support the view that unsaponifiable matter is synthesized in the body, because it continued to be excreted though none was in the food; but the data also present some phases which cause us to question whether it reaches the intestine by the way of the bile. Because of the constant relationships between unsaponifiable material and fatty acids shown throughout the work, it seems improbable that they come from different sources; but what evidence has been obtained so far indicates that the fecal fatty acids are ordinary, usable fatty acids, which, if excreted by way of the bile, would be reabsorbed. It is true that normal oleic acid has not been definitely identified and it is possible that the liquid fraction is made up of isomeric acids with the unsaturated groups in such positions that the acids cannot be used by the organism; but, in view of the fact that the body seems able to handle any monobasic straight chain acid, even with an odd number of carbon atoms, this does not seem very possible. It seems quite definite that the solid acids consist at least largely, if not entirely, of usable stearic and palmitic. The possibility

that the fatty acids are bound to the sterols as esters and thus escape absorption is largely ruled out by the experiment in which the distribution of the lipids was studied (Lipid-free 4), where it was shown that approximately 70 per cent of the fatty acids were present in the form of free acids or soaps and that there was little, if any, difference in the composition of the free and combined acids. The same thing is of course shown in a general way by the finding of ratios of unsaponifiable to fatty acids of about 0.45 throughout the work while in cholesterol esters the ratio is about 1.5. It has seemed possible that the bile might be either eliminated or designated as the source of lipid excretion by experiments similar to those above in which animals with bile fistulas were fed a lipid-free diet. Such experiments are now being carried out and, though not enough data have been collected to warrant the drawing of conclusions, it may be stated that the results so far indicate that more lipids are excreted by dogs under the conditions described than normally. It seems rather probable, therefore, that the lipids making up the basal excretion with lipid-free diets reach the intestine through its walls below the absorbing portion.

The purpose of the excretion is as yet unknown. As pointed out in the preceding paper there is a marked similarity in the composition of the fecal lipids and those of blood plasma. In only one point is there a great difference noted, *i.e.* the liquid acids of the plasma are considerably more unsaturated than those of the feces; but this may be explained as being due to the known reducing action of the intestine. This similarity suggests that there is some sort of leakage of plasma lipids into the intestine, though why such a leakage should occur in view of the general absence of such phenomena is difficult to see. If such a leakage occurred, it might explain, however, the increased excretion when lipids were included in the food and the similarity between the lipids making up the increase and the diet lipids, assuming, as mentioned above, a plethora of lipids similar to those of the diet. The possibility has been suggested that this may be a leakage designed to lubricate the interior surface of the intestine.

There is still another possibility, which was suggested by recent work of Gardner (23) in which he separated the unsaponifiable matter of human feces and found that little over half formed

insoluble digitonides, the remainder being made up of substances marked by great stability, containing oxygen in a not very reactive form, and having a molecular composition of much the same order as cholesterol. It has been noted in this investigation that the unsaponifiable fraction was usually obtained as a glassy, non-crystalline material answering the description of some of the fractions obtained by Gardner and it is suggested that these substances may be waste products of cholesterol metabolism and the lipid excretion concerned with removing them from the body, the fatty acids and neutral fats acting in some manner, perhaps as solvent, to carry them across the intestinal wall. In favor of such a view is the tendency toward a constant excretion of unsaponifiable material, shown particularly by the high proportional excretion of this fraction during fasting, where the amounts excreted were of the same order as in Lipid-free 3 though the total lipid excretion was considerably smaller. It is noticeable also that the ratio of liquid to solid acids is much higher in the case of the fasting animals than with a lipid-free diet and it is suggested as a working hypothesis that there may be a certain optimum mixture of fats for carrying the undesirable sterols across the intestinal wall in solution; but that during fasting, when the body is attempting to conserve all of its resources, it is possible for it to carry on this process of excretion with less expenditure of useful fat by employing a higher proportion of liquid acids.

SUMMARY.

1. Dogs between 5 and 13 kilos in weight excrete an average of 1.5 to 2.0 gm. of non-volatile lipids per week on a lipid-free diet, this basal excretion being made up of 35 to 40 per cent unsaponifiable material and 55 to 60 per cent non-volatile acids, which contain about 30 per cent solid and 60 per cent liquid acids. The solid fraction is composed of palmitic and stearic acids with the stearic present in slightly larger amount, while the liquid fraction consists almost entirely of an acid with one double bond, probably oleic, together with small amounts of an acid with four double bonds, most likely arachidonic.

2. Lipids of the composition given above continue to be excreted after 5 weeks without lipids in the diet, though there is a slightly smaller amount excreted at the end than at the beginning of the experiment.

3. In one experiment with a lipid-free diet, where the feces were extracted without preliminary saponification, it was found that the fecal lipids consisted of about 40 per cent free fatty acids (including soaps) and 60 per cent neutral material. About 75 per cent of the neutral fraction was unsaponifiable while the remaining 25 per cent consisted of fatty acids. Both the free and the combined fatty acids were of essentially the same composition as described above.

4. Volatile acids have been determined by the Gillespie and Walters method. The average composition has been found to be approximately 65 per cent acetic, 23 per cent butyric, and 12 per cent caproic acids.

5. The composition of the lipids excreted by different dogs under the same conditions has been found in most cases to be remarkably uniform, though there has been considerable variation in the amount.

6. The source and purpose of lipid excretion is discussed. The presence of usable fatty acids not bound to sterols makes it probable that fecal lipids enter the intestine through its walls below the absorbing portion rather than through the bile. The similarity to plasma lipids indicates that they represent some sort of a leakage and the suggestion has been made that the leakage has the purpose of lubricating the intestine. On the other hand some evidence has been presented in favor of the view that the lipid excretion is concerned with removing undesirable or excess sterols from the organism. Further work is being carried on in an attempt to settle more definitely the purpose of lipid excretion.

The author wishes to acknowledge his indebtedness to Professor W. R. Bloor for suggesting the problem of this investigation and for his continued helpful advice throughout the work.

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ANIMAL CALORIMETRY.

THIRTY-FIRST PAPER.

RESPIRATORY METABOLISM IN PHLORHIZIN DIABETES AFTER GLUCOSE INGESTION.

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In the first paper published upon the D:N ratio in phlorhizinized dogs (1) it was calculated by Reilly, Nolan, and Lusk that when 24 gm. of glucose were given *per os* to a completely phlorhizinized dog 22.49 gm. (93.7 per cent) were recovered in the urine as "extra sugar." A carefully performed experiment by A. I. Ringer (2) showed that when 75 gm. of glucose were given *per os* in six doses to a dog 69.5 gm. (93 per cent) were eliminated unoxidized as extra sugar in the urine. The D:N ratios of the days preceding and following the ingestion of this amount of glucose were 3.62 and 3.64, respectively, which gave the right to consider that the dog was completely phlorhizinized and that the work was accomplished under the original conditions laid down by Lusk. In Ringer's experiment the nitrogen in the urine, which had been 14.0 gm. the day before the experiment, declined to 9.3 gm. on the day of sugar ingestion and returned to 14.4 gm. on the following day. Ringer concluded that the administration of glucose to phlorhizinized dogs resulted in the sparing of protein, though none of the glucose was oxidized.

Lusk (3) found a respiratory quotient of 0.716 in a dog during a period of fasting and phlorhizin influence and an average quotient of 0.711 in the 2nd and 3rd hours immediately following the ingestion of 10 gm. of glucose. He found an average respiratory quotient of 0.70 following the ingestion of 10 gm. of fructose in the phlorhizinized dog and one of 0.724 in the same dog during the 2nd hour after giving 70 gm. of glucose. The D:N ratio during the last named period rose to 23:1, showing a large movement of sugar molecules. Lusk stated, "The respiratory quotient indicates that glucose could have been oxidized only in minimal quantities."

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Michael Ringer (4) gave 40 gm. of glucose to a completely phlorhizinized dog and recovered 36.4 gm. as extra glucose in the urine, or 91 per cent. The basal quotient was 0.711 and after giving the glucose it averaged 0.711 per hour for 3 hours. Gaebler and Murlin (5) confirm the work.

The validity of the almost quantitative elimination of extra sugar in hourly periods in the experiments of Csonka (6) and of Deuel and Chambers (7) depends upon the principle that glucose is not oxidized, as does also the measurement of the quantity of sugar arising from amino acids, as determined by Lusk (8), Ringer and Lusk (9), Warkalla, in Cremer's laboratory (10), and by Dakin (11).

Deuel and Chambers noticed a great improvement in the physical power of their phlorhizinized dogs after giving them glucose, and they were the first to follow the urinary nitrogen by hourly periods. The protein metabolism gradually fell until the 7th hour and then gradually rose again. In agreement with Ringer, they concluded that the effects of the administration of glucose were not due to its oxidation.

S. R. Benedict and Osterberg (12) have reported that if meat is given to a phlorhizinized dog the acetonuria may completely disappear, the D:N ratio remaining constant. This was not true of Benedict and Lewis' case (13) of human carcinoma, who was completely phlorhizinized. Neither meat nor glucose ingestion reduced the acidosis in this man.[1]

My own particular interest has been the study of acetonuria in phlorhizin glycosuria in dogs, concerning which a preliminary report (14) has already been published from this laboratory.[2]

Sansum and Woodyatt (15) have reported that, after giving

[1] Personal communication to Professor Lusk.

[2] On account of some fundamental differences of opinion with regard to the interpretation of this work it has been agreed that Dr. Wierzuchowski, without prejudice to himself, publish the full report of his results in such form and place as he desires and without the supervisory control of this laboratory. This is because it does not seem possible to me that the frequent, nearly complete recovery of small amounts of ingested glucose, alanine, and glycine in the form of "extra glucose" in the urine is the result of the manipulation of figures to suit my purpose. It may, however, be added that the recovery of *i*-alanine as extra sugar has frequently been less than the theory called for, and so for many years (Csonka, F. A., *J. Biol. Chem.*, 1915, xx, 539) it has been assumed in this laboratory that there is an average recovery of extra sugar from alanine of only 70 per cent. Urinary analysis (Lusk, G., *J. Biol. Chem.*, 1912–13, xiii, 155) indicates that at times some alanine may be excreted unchanged in the urine. When alanine, the respiratory quotient of which is 0.83, is administered to a fasting and completely phlorhizinized dog (Lusk, G., *J. Biol. Chem.*, 1915, xx, 555) it fails to increase the usual diabetic respiratory quotient.

Graham Lusk.

8 to 16 gm. of glucose to phlorhizinized dogs, the recovery of extra sugar varies between 50 to 86 per cent.

Nash (16), in a recent paper, reports an average recovery of 90 per cent. My own results, as calculated by me show a recovery of only 80 per cent. It seemed therefore probable that the complete abolition of the acidosis of the phlorhizinized dog after the ingestion of glucose, which I have noted, is in reality due to the oxidation of a part of the ingested glucose. This could be determined only by measurements of the respiratory quotients of the dog. To this end a new series of experiments was carried out in order to determine from the respiratory quotients the possible intensity of the oxidation of carbohydrate.

Methods.

The usual methods of calorimeter technique employed in this laboratory were adopted. Phlorhizin, 1 gm., was rubbed in sterile olive oil and was injected once daily. The basis of the calculations of the respiratory metabolism when an animal is diabetic has been described elsewhere (3).

Results.

The *basal metabolism* of three fasting phlorhizinized dogs showed as constant respiratory quotients as those obtained when alcohol was burned in the calorimeter.

Dog No.	Date.	R. Q.	Alcohol checks.		
			No.	Date.	R. Q.
	1926			*1926*	
42	Feb. 2	0.699	207	Jan. 26	0.668
43	" 8	0.697	208	Feb. 15	0.670
44	" 16	0.701	209	" 19	0.645
44	" 26	0.702	210	" 20	0.653
			211	" 24	0.669
			212	Mar. 19	0.666

Theoretical R. Q. for alcohol = 0.667.

Administration of 20 Gm. of Glucose.—The method followed was twofold. In three experiments 20 gm. of glucose were ad-

ministered just before the animal was introduced into the calorimeter, and in two experiments 20 gm. were given followed 4 hours later by a second administration of 20 gm. of glucose. This procedure was followed because 4 hours after the ingestion of glucose the ketosis has disappeared and the CO_2-combining power of the blood has been raised to the normal. Actual experiment has shown that under these conditions a second administration of glucose does not change the CO_2-combining power of the blood during the period corresponding to that of the calorimeter experiment (see Table V).

A discussion of the diabetic respiratory quotient has been published elsewhere by Lusk (17). At first sight it might appear that, after giving glucose to the phlorhizinized dog, the CO_2 retention by the blood through the rise in the CO_2-combining power of the blood during the process of abolishing the condition of acidosis might be sufficient to depress the quotient and to mask the effect of a true oxidation of glucose. It should be remembered that when 36 gm. of β-oxybutyric acid (R. Q. = 0.889) are formed from 100 gm. of fat (R. Q. = 0.707), there may be 15.23 gm. of CO_2 liberated from $NaHCO_3$ so that the resulting diabetic quotient for fat may be calculated at 0.715. Conversely, when 36 gm. of β-oxybutyric acid are oxidized, the alkali liberated might conceivably be sufficient to unite with 15.23 gm. of CO_2. The respiratory quotient of this reaction would be 0.667. It is therefore apparent that there is a theoretical possibility that, during the time that acidosis is being abolished, an effect is produced which may somewhat mask the effect of an oxidation of glucose. Hence, the preliminary dose of glucose which abolishes the acidosis and establishes the CO_2-combining power of the blood at a normal level leads to results of unquestionable accuracy during the calorimeter periods of the hours following the second administration of glucose.

Table I shows that after giving 20 gm. of glucose to two phlorhizinized dogs, such weighing less than 10 kilos, the respiratory quotients may rise during the immediately succeeding hours above the basal level of 0.700, but that the original basic level returns after 2 or 3 hours. Unfortunately, no urinary analyses were made upon Dog 43, so that a close examination of the data is possible only in the case of Dog 44.

One may compute from the non-protein respiratory quotients the quantity of glucose oxidized during the various periods, and these data are presented in Table II. On the 6th day of diabetes it may be calculated that after 20 gm. of glucose were ingested

TABLE I.

Showing the Influence of the Ingestion of 20 Gm. of Glucose upon the Respiratory Quotients in Two Phlorhizinized Dogs.

	Dog No.	Experiment No.	Date.	Body weight.	Approximate hr. after last glucose.							Time between glucose ingestion and start of experiment.
					1	2	3	4	5	6	7	
			1926	*kg.*								*min.*
20 gm. glucose.....	43	2	Feb. 9	8.85			0.723	0.703	0.703	0.709	0.706	111
20 " "	43	3	" 10	8.70		0.735*	0.735*	0.700*	0.700*	0.700*		60
20 " "	44	2	" 17	7.40	0.756	0.750	0.702*	0.702*	0.702*			16
20 " " (two doses 4 hrs. apart)	44	3	" 18	7.20	0.801	0.741	0.768	0.710*	0.710*			25
20 " " " " 4 " "	44	5	" 26	6.35	0.771	0.802	0.702*	0.702*				28

* Averages of 2 or 3 successive hours.

TABLE II.

Showing the Non-Protein Respiratory Quotients and Calculated Sugar Oxidation during Successive Hours in Dog 44, When Phlorhizinized and Fasting or Following Glucose Ingestion in a Single Dose or after Double Dosage 4 Hours Apart.

Date.	Days since first phlorhizin.	Experiment No.		1st hr.	2nd hr.	3rd hr.	4th hr.	5th hr.	Total glucose oxidized.
1926									*gm.*
Feb. 16	5	1	Basal.	0.717	0.717				
" 17	6	2	Glucose, 20 gm.	0.788	0.788	0.721	0.721	0.721	
				1.15 gm.	1.15 gm.				2.30
" 18	7	3	" 20 " twice.	0.820	0.753	0.783	0.718	0.718	
				2.27 gm.*	0.72 gm.	1.09 gm.			4.08
" 26	15	4	Basal.	0.714	0.714				
" 27	16	5	Glucose, 20 gm. twice.	0.807	0.807	0.711	0.711		
				1.40 gm.	1.40 gm.				2.80

* 1½ hours.

2.3 gm. were oxidized during the 1st and 2nd hours, or 11.5 per cent.

After two successive administrations of glucose in doses of 20 gm. 4 hours apart an oxidation of 4.08 gm. occurred during the first 3 hours following the experiment on the 7th day of diabetes, and one of 2.80 gm. on the 16th day of diabetes. This means

TABLE III.

Showing a Calculation of the Mixture of the Metabolites Oxidized per Hour by Dog 44, Phlorhizinized: Hours Following Glucose Ingestion.

	Experiment 2. Average 2 hrs.		Experiment 3. Average 3 hrs., 20 min.		Experiment 5. Average 2 hrs.	
	cals.	*gm.*	*cals.*	*gm.*	*cals.*	*gm.*
Protein	3.81	1.83	1.46	0.70	1.46	0.70
Fat	10.12	1.07	11.63	1.22	8.01	0.84
Glucose	4.32	1.15	4.64	1.24	5.25	1.40
	18.25		17.73		14.72	
$\frac{\text{Fatty acid}}{\text{Glucose}}$ (Woodyatt)		1.40		1.00		0.72
$\frac{\text{Ketogenic mols}}{\text{Glucose mols}}$ (Shaffer)		1.15		0.80		0.55
Shaffer from R. Q		0.60		0.80		0.44
R. Q. of dog		0.79		0.77		0.81

TABLE IV.

Total Metabolism per Hour of Dog 44.

	Feb. 16. Basal.	Feb. 17. Glucose, 20 gm.		Feb. 18. Glucose, 20 gm. + 20 gm.		Feb. 25. Basal.	Feb. 26. Glucose, 20 gm. + 20 gm.	
	II 2 hrs.	I 2 hrs.	II 2 hrs.	I 3½ hrs.	II 2 hrs.	II 2 hrs.	I 2 hrs.	II 2 hrs.
Calories, indirect	19.54	18.25	18.87	17.73	17.72	16.44	14.72	16.27
" eliminated*	17.88	14.97	18.24	17.46	17.91	14.69	12.50	15.84

* Rectal thermometer not used.

I represents an elevated R. Q. period.

II represents periods with the fasting diabetic R. Q.

that, of 20 gm. given in the second dose, 20 and 14 per cent of the material given was oxidized.

These results throw some doubt on the validity of the conclusions of Michael Ringer (4) on the influence of insulin on phlorhizin

TABLE V.

Effect of Glucose on Urine and CO_2-Combining Power of Blood.

Dog 44, phlorhizinized since Feb. 11, 1926. On Feb. 27, body weight 6.15 kilos.

Date and time.	Urine.								Blood CO_2-combining power.	Remarks.
	Nitrogen.		Sugar.		D:N	Acetone.	Diacetic acid.	Ketone bodies in breath.		
	Total.	Per hr.	Total.	Per hr.						
1926	*gm.*	*gm.*	*gm.*	*gm.*					*vol. per cent*	
Feb. 25	0.656	0.182	2.55	0.71	3.89					Basal metabolism.
" 26										
6.00 a.m.										20 gm. of glucose by stomach tube.
10.00 "										Calorimeter experiment. 20 gm. of glucose by stomach tube.
Feb. 27										
11.00 a.m.– 3.00 p.m.	0.587	0.147	2.43	0.61	4.14	+	+	+	47.5	Some sugar from preceding day is still excreted.
3.00 p.m.									2.30 p.m.	
3.00 " – 7.00 "	0.759	0.190	11.50	2.88	15.16	+	+	+	57.1	During this time calorimeter experiment was made on Feb. 26.
7.00 "									6.53 p.m.	
7.00 " –11.23 "	0.492	0.112	12.03	2.75	24.47	0	0	0	59.0	
									11.20 p.m.	
11.23 " – 3.06 a.m.	0.362	0.097	2.45	0.66	6.78	0	0	0	58.5	Calculation is made on basis of D:N of
									3.00 a.m.	3.89 from Feb. 25.
Feb. 28										
3.06 a.m.– 3.48 p.m.	1.373	0.108	10.08	0.79	7.35	0	0	0		Total sugar. 48.57
3.48 p.m.– 8.51 "	0.634	0.126	4.15	0.82	6.55	0	0	0		Protein " −21.78
8.51 " – 9.15 a.m.	1.965	0.159	8.36	0.67	4.26	+	0	0		26.79 = 66.97 per cent recovery of sugar administered.
Total 42 hrs., 15 min.	5.585		48.57							

diabetes published from this laboratory, work which has been criticized both by Nash (18) and by Colwell (19), but which has been confirmed by Gaebler and Murlin (5). Michael Ringer's work may be thus epitomized:

	Length of experiment.	R. Q.
	hrs.	
Phlorhizin, basal, fasting	3	0.711
Glucose, 40 gm.	4	0.711
" 40 " + insulin	4	0.736
" 20 " + " 3 hrs. before, and 40 gm. + insulin 1 hr. before	4	0.823

Although at first the barrier against the combustion of glucose was complete, the later experiments accord with my own results except that the oxidation of the glucose administered simultaneously with insulin in Ringer's experiment lasted much longer than in my own experiments without insulin.

TABLE VI.

Alcohol Checks.

Date.	Experiment No.	Length.	Calories.		R. Q.
			Calculated.	Found	
1926		*hrs.*			
Jan. 26	207	5	16.70	17.32	0.668
Feb 15	208	4	17.60	15.90	0.670
" 19	209	2	27.43	29.93	0.645*
" 20	210	4	28.41	29.01	0.653*
" 24	211	4	16.35	16.79	0.669
Mar. 19	212	1	28.00	28.11	0.667
Average			22.42	22.84	

* The reason for these low values is unknown.

It is possible to compute from my own work the mixture of materials which underwent destruction in the phlorhizinized dogs after glucose ingestion and to compute the ratio $\frac{\text{gm. of fatty acid}}{\text{gm. of glucose}}$ of Woodyatt (20) and the ratio $\frac{\text{millimols of acetoacetic acid}}{\text{millimols of glucose}}$ of

TABLE VII.

Calorimeter Experiments. Values Given as Averages per Hour.

Date.	Experiment No.	Time.	CO_2	O_2	R.Q.	H_2O	Urine N.	Non-protein. CO_2	Non-protein. O_2	Non-protein. R.Q.	Calories. Protein.	Calories. Non-protein.	Calories. Indirect.	Calories. Eliminated.	Urine. Sugar per hr.	Urine. D:N	Urine. Ketones.	Morning weight.	Diet.
1926			*gm.*	*gm.*		*gm.*	*gm.*	*gm.*	*gm.*									*kg.*	
Dog. 42.																			
Feb. 2	1	1.12 p.m.–3.12 p.m.	5.17	5.38	0.699									18.02				6.85	4th day phlorhizin. Basal metabolism. Semicomatose. Died next day.
Dog 43.																			
Feb. 8	1	1.15 p.m.–3.15 p.m.	8.23	8.36	0.697									23.74				9.40	4th phlorhizin day. Basal. No coma. Glucose, 20 gm. in 100 cc. water at 9.18 a.m.
" 9	2	11.09 a.m.–4.11 p.m.	8.56	8.76	0.711									26.70				8.85	
" 10	3	10.28 a.m.–12.28 p.m.	8.02	7.93	0.735									22.69				8.70	Glucose, 20 gm. in 100 cc. water at 9.28 a.m. CO_2-combining power: 9 a.m., 45 per cent; 4 p.m., 55 per cent.
		12.28 p.m.–3.38 p.m.	7.63	7.94	0.700									24.40					

Dog 44.																			
Feb. 16	1	11.53 a.m.–1.53 p.m.	5.88	6.09	0.701	6.10	0.255	4.86	4.93	0.717	3.33	16.21	19.54	17.88	0.89	3.47	+++	7.60	5th day phlorhizin. Basal.
" 17	2	10.21 a.m.–12.21 p.m.	5.84	5.64	0.753	5.10	0.292	4.67	4.31	0.788	3.81	14.44	18.85	14.97	2.60	8.90	Slight.	7.40	Glucose, 20 gm. at 10.05 a.m. D:N = 3.53 in urine preceding the experiment.
		12.21 p.m.–3.21 p.m.	5.71	5.91	0.702	5.55	0.292	4.54	4.58	0.721	3.81	15.07	18.87	18.24	2.60	8.90			
" 18	3	10.58 a.m.–12.18 p.m.	5.74	5.21	0.801	6.73	0.112	5.29	4.69	0.820	1.46	15.84	17.30	16.17			0	7.20	Glucose, 20 gm. at 6 a.m. Same at 10 a.m.
		12.18 p.m.–1.18 p.m.	5.80	5.69	0.741	7.97	0.112	5.35	5.17	0.753	1.46	17.15	18.61	17.93					
		1.18 p.m.–2.18 p.m.	5.48	5.19	0.768	7.54	0.112	5.03	4.67	0.783	1.46	15.61	17.07	18.29					
		2.18 p.m.–4.18 p.m.	5.33	5.46	0.710	8.05	0.112	4.88	4.94	0.718	1.46	16.26	17.72	17.91					
" 25	4	1.25 p.m.–3.25 p.m.	4.93	5.11	0.702	7.96	0.182	4.20	4.28	0.714	2.37	14.07	16.44	14.69	0.71	3.89	+++	6.40	14th day phlorhizin. Basal.
" 26	5	10.28 a.m.–12.28 p.m.	4.82	4.46	0.787	6.25	0.112	4.37	3.94	0.807	1.46	13.26	14.72	12.50	2.75	24.47	0	6.35	Glucose, 20 gm. at 6 a.m. Same at 10 a.m. See Table V for special results.
		12.28 p.m.–2.28 p.m.	4.86	5.03	0.702	6.35	0.112	4.41	4.51	0.711	1.46	14.81	16.27	15.84	2.75				

Shaffer (21). To these calculations has been added the calculation based on the respiratory quotient alone, as given by Shaffer (22).

Table III expresses these results.

It is obvious that the metabolic mixture of these periods was such that in each case acidosis would be prevented during the periods following the ingestion of glucose. Therefore, the acidosis disappears, protein metabolism falls, and the physical power of the animal revives.

Total Metabolism.—There was no distinct effect upon the height of the metabolism during the short period when glucose was oxidized, as compared with that of the basal metabolism or of the hours immediately following glucose oxidation. This appears in the above compilation (Table IV).

The Urine and Blood.—After the completion of the last calorimeter experiment two successive doses of glucose were given to the dog, as on the previous day of calorimeter work, in order to determine the course of the acidosis, as indicated by the CO_2-combining power of the blood. The details of this work are given in Table V.

It is obvious from this table that by no method of computation is it possible to show a complete recovery of ingested glucose in the urine. A part of this discrepancy is certainly due to the oxidation of the glucose by this phlorhizinized dog.

A record of the alcohol checks is submitted in Table VI and a summary of the results with the calorimeter in Table VII.

SUMMARY.

It has been found that *some* phlorhizinized dogs may oxidize small amounts of glucose for 2 or 3 hours after 20 gm. of the material is ingested. This was especially marked after 20 gm. of glucose were ingested in two successive doses 4 hours apart. Under these circumstances the respiratory quotient has been seen to rise from the diabetic level of 0.700 to 0.80. The calculated oxidation of glucose in two experiments on one dog represented 20 and 14 per cent of the 20 gm. ingested in the second dose. This offers an explanation of the disappearance of the acidosis. In this dog, however, the recovery of ingested glucose as extra

glucose in the urine was far from complete, and the results, therefore, do not contradict other carefully executed work done in this laboratory which shows that in other phlorhizinized dogs there may be little or no oxidation of ingested glucose.

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SCYLLITOL FROM FLOWERING DOGWOOD (CORNUS FLORIDA).

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Scyllitol, $C_6H_6(OH)_6$, was first isolated in 1858 by Staedeler and Frerichs[1] from the kidneys and other organs of certain plagiostomous fishes. It has since been found in acorns,[2,3] in the leaves of *Cocos plumosa* and *Cocos nucifera*,[4] and in the leaves of *Helinus ovatus*.[5] As a result of the present investigation, the flowering dogwood, *Cornus florida*, may be added as another source of the compound.

EXPERIMENTAL.

The material employed in this investigation consisted of the flowers and bracts of flowering dogwood collected in April near Washington, D. C. The total quantity of material collected was 79.6 kilos, which when air-dried amounted to 16.3 kilos. The entire quantity was coarsely ground and extracted, first by continuous percolation with ether to remove fats and other substances, and finally with hot redistilled 95 per cent alcohol. After concentrating the alcoholic extract, it was set aside in a cool place for several months, whereupon a dark semicrystalline material gradually separated out. This was collected by filtration, washed with alcohol, and dried in the air. The crude material was dissolved in boiling water, filtered from a small quantity of dark insoluble substance, and heated several times with animal charcoal

[1] Staedeler, G., and Frerichs, F. T., *J. prakt. Chem.*, 1858, liii, 48.
[2] Vincent, C., and Delachanal, *Compt. rend. Acad.*, 1887, civ, 1855.
[3] Müller, H., *J. Chem. Soc.*, 1912, ci, 2383.
[4] Müller, H., *J. Chem. Soc.*, 1907, xci, 1767.
[5] Goodson, J. A., *J. Chem. Soc.*, 1920, cxvii, 140.

to remove most of the color. The solution was then concentrated on the steam bath to a small volume. Transparent, granular, crystalline aggregates separated out on standing. Freed from the mother liquor these appeared to be contaminated with inositol. Further purification was accomplished by dissolving in hot water and repeating the foregoing process.

At the end of the treatment, the substance lacked the characteristic sweetness of inositol, and had a much higher melting point. It amounted to nearly 2 gm., air-dried. In order to eliminate final traces of inositol, the dried substance was pulverized, washed with a little cold water, and recrystallized several times by dissolving in boiling water, filtering, and precipitating with hot 95 per cent alcohol. The best method of obtaining well defined crystals is to evaporate slowly an aqueous solution on the steam bath. Crystals form on cooling or during the evaporation when the proper concentration is reached.

The substance thus obtained forms colorless, glistening, well defined monoclinic rhombs. It is not very soluble in cold water but is fairly soluble in boiling water; it is only very slightly soluble in cold alcohol and practically insoluble in ether. When heated the compound gradually darkens at 315°. Above 335° it becomes so dark that its melting point cannot be determined. After being dried at 120–125°, it was analyzed with the following results.

0.1100 gm. gave 0.1617 gm. CO_2 and 0.0685 gm. H_2O.
Found. C 40.09, H 6.98.
$C_6H_{12}O_6$ requires: C 39.98, H 6.72.

These results, together with the properties just described, suggest that the compound is identical with scyllitol. In order to confirm this, the substance was converted by acetylation into an acetyl derivative by heating for 6 hours with acetic anhydride and anhydrous sodium acetate. The reaction proceeded very slowly, gradual solution occurring with the separation of the sparingly soluble, glistening, acetyl derivative. When the reaction appeared to be complete the mass was poured into water and set aside overnight. The deposit was then collected on a Büchner funnel, washed with water, and crystallized from hot glacial acetic acid, from which it separated on cooling as glistening prismatic crystals. Its melting point was 291°, which agrees

with that found by Müller for hexacetyl scyllitol. The derivative was thoroughly washed with water to remove acetic acid and then dried overnight at 120°. Combustion gave the following results.

0.1648 gm. gave 0.3020 gm. CO_2 and 0.0838 gm. H_2O.
0.1489 " " 0.2731 " " " 0.0754 " "
Found. C 49.97, 50.02; H 5.70, 5.66.
$C_6H_6O_6(CO \cdot CH_3)_6$ requires: C 49.98, H 5.60.

In view of the excellent agreement of the analytical results with the figures for scyllitol and its hexacetyl derivative, complete identification required only the determination of crystallographic and optical properties.

Crystals of the original substance were mounted upon the two-circle Goldschmidt goniometer, and the reflections from the crystal faces were observed. In every case the ϕ and ρ values for the angles agreed within experimental error with those measured for scyllitol by Barker.[3] It was noted, however, that the form r $(\bar{2}01)$ did not occur upon these crystals. No significance need be attached to this fact, as a single form is sometimes suppressed on account of the environment under which a compound may crystallize.

The optical properties also confirm the identity of our compound with scyllitol. The extinction angle on m is 33° with the edge mm, this value being in exact agreement with previous observations.[4] A complete study of the substance under the petrographic microscope was undertaken.[6] In ordinary light the habit was not characteristic, broken angular fragments being usually present. These showed the indices $\alpha = 1.570$, $\beta = 1.580$, and $\gamma = 1.610$, $\gamma - \alpha = 0.040$, all ± 0.001. In plane polarized light with crossed nicols the birefringence is strong, colors of the first and second orders being observed on thin grains while third order colors are common on thicker grains. In convergent polarized light with crossed nicols a biaxial interference figure is observed showing one optic axis. The axial angle is large and the optic sign is positive.

Our hexacetyl scyllitol was not measured on the goniometer, but the extinction angle observed under the microscope agreed with that previously recorded. The following additional optical

[6] Wherry, E. T., *U. S. Dept. Agric. Bull. 679*, 1918.

properties were observed. In ordinary light the compound showed terminated needle-like crystals of characteristic habit. The refractive indices at 20° were $\alpha = 1.480$ (crosswise on needles), β was indeterminable, but a mean value of β and $\gamma = 1.510$ often occurred and is diagnostic for this substance, $\gamma = 1.515$ (lengthwise on needles), $\gamma - \alpha = 0.035$, all ± 0.001. The sign of elongation is positive. In plane polarized light with crossed nicols the crystals are anisotropic, extinction on edge *mm* is sharp and equal to 33°, birefringence is medium, and colors of first and second orders are common. When γ is up the extinction is probably parallel, but this condition is rare. In convergent polarized light with crossed nicols a biaxial interference figure showing one optic axis up, but inclined, is sometimes observed. The optic sign is negative and the axial angle is much smaller than that of scyllitol, probably about 30 to 40°.

The foregoing data completely confirm the identity of the compound isolated from the flowering dogwood with scyllitol.

CONCLUSION.

Scyllitol, $C_6H_6(OH)_6$, an isomer of inositol, has been isolated from the flowers and bracts of flowering dogwood, *Cornus florida*. It was identified by combustion of the purified substance and of its hexacetyl derivative, and also by means of crystallographic and optical properties.

INOSITOL FROM BLACKBERRY (RUBUS ARGUTUS LINK) AND FLOWERING DOGWOOD (CORNUS FLORIDA).

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In the course of a study of the flavonol coloring matters from the tall blackberry (*Rubus argutus* Link) and the flowering dogwood (*Cornus florida*) a substance identified as inositol was isolated from these plants. In the belief that every definitely established occurrence of a plant product is worth recording, I present this slight addition to our knowledge of the distribution of inositol in plants.

Inositol from Blackberry.

The blackberry material employed in this investigation consisted of leaves collected in July near Washington, D. C. The entire quantity of air-dried material, amounting to 3.1 kilos, was coarsely ground and extracted first with ether to remove fats, plastid pigments, and other impurities, and then with hot redistilled 95 per cent alcohol. After concentrating the alcoholic extract, it was set aside in a cool place for several months whereupon a semi-crystalline material gradually separated. This was collected on a Büchner funnel, and washed with cold 95 per cent alcohol. The crude substance was dissolved in cold water and filtered from a small quantity of dark insoluble impurity. The solution was then heated with animal charcoal to remove most of the color. From its solution in water, the substance separated in a crystalline state upon the slow addition of four volumes of hot 95 per cent alcohol. The foregoing process of purifying by solution in water and precipitation with alcohol was repeated several times. The compound was recrystallized from boiling 95 per cent alcohol containing sufficient water to insure solution. When air-dried, it amounted to 4.8 gm., thus representing 0.15 per cent of the air-

dried weight of blackberry leaves. Final purification was accomplished by solution in a small quantity of water and addition of hot 95 per cent alcohol. More complete separation of the compound from its dilute alcoholic solution was effected by adding one-tenth the volume of ether.

The substance, obtained in this manner, consisted of colorless, glistening crystals which were easily soluble in cold water, sparingly soluble in alcohol, and almost insoluble in ether. The compound possessed a characteristic sweet taste and gave a melting point of 224–225°. Analysis of the substance, dried at 120°, showed that it agreed in composition with inositol.

0.1387 gm.gave 0.2033 gm. CO_2 and 0.0805 gm. H_2O.
0.1204 " " 0.1767 " " " 0.0716 " "
Found. C 39.90, 40.02; H 6.51, 6.67.
Inositol, $C_6H_{12}O_6$, requires: C 39.98, H 6.72.

The identity of this compound with inositol was confirmed by the formation of its acetyl derivative, which was prepared by boiling the substance with acetic anhydride and anhydrous sodium acetate for 1 hour. The crude derivative obtained by adding water to the reaction mixture was purified by several crystallizations from boiling 70 per cent alcohol. It separated as colorless, tabular crystals closely resembling pieces of mica. These were insoluble in water and sparingly soluble in cold 70 per cent alcohol. They melted at 214–215°. In some textbooks (*e.g.*, Richter) the melting point of hexacetyl inositol is given as 211°, but a more recent determination by Miyake[1] shows it to be 215°. With this value mine is in excellent agreement. Analysis of the derivative, dried at 110°, showed it to be identical in composition with hexacetyl inositol.

0.1270 gm. gave 0.2315 gm. CO_2 and 0.0613 gm. H_2O.
0.1569 " " 0.2885 " " " 0.0751 " "
Found. C 49.71, 50.11; H 5.41, 5.36.
Hexacetyl inositol, $C_6H_6O_6(CO \cdot CH_3)_6$, requires: C 49.98, H 5.60.

Inositol from Flowering Dogwood.

The dogwood material employed for this work consisted of the flowers and bracts collected in April near Washington, D. C. The

[1] Miyake, S., Chemical studies of corn pollen. I. Isolation of phytosterol and inositol, *J. Biochem.*, Japan, 1922, ii, 27.

coarsely ground, air-dried material, amounting to 16.3 kilos, was extracted with ether to remove impurities, and then with 95 per cent alcohol. The concentrated alcoholic extract deposited, on long standing, a semicrystalline substance which appeared to consist of two products, one more soluble in water than the other. The less soluble substance proved to be scyllitol.[2] The second product was obtained by treating the aqueous mother liquors from which the scyllitol had been separated with successive portions of hot 95 per cent alcohol. The first separation was mixed with a trace of scyllitol and was, therefore, discarded. The later deposits, obtained on further additions of alcohol, were purified by repeated crystallizations from dilute alcohol. They proved to be identical and, combined, amounted to nearly 6 gm., air-dried. Finally, the entire quantity was dissolved in a little cold water, filtered, and treated slowly with hot 95 per cent alcohol. On standing, colorless, glistening, crystals were deposited which melted at 224–225° and corresponded in their other characteristics with inositol. When mixed with authentic inositol, there was no depression of the melting point. Combustions of the substance, dried at 110–115°, yielded the following results.

0.1207 gm. gave 0.1777 gm. CO_2 and 0.0712 gm. H_2O.
0.1062 " " 0.1551 " " " 0.0658 " "
0.1032 " " 0.1510 " " " 0.0610 " "
Found. C 40.15, 39.83, 39.90; H 6.61, 6.94, 6.62.
Inositol, $C_6H_{12}O_6$, requires: C 39.98, H 6.72.

In view of the close agreement of these results with the figures for inositol, complete identification required only the preparation and analysis of the acetyl derivative. This was prepared in the usual manner by the use of acetic anhydride and anhydrous sodium acetate and crystallized from 70 per cent alcohol. The results of analyses indicated incomplete acetylization. The substance was consequently heated another ½ hour with acetic anhydride alone. Separation of the acetyl product was effected by the addition of 95 per cent alcohol. This was collected and crystallized from 70 per cent alcohol and finally from 50 per cent alcohol. It was deposited as scaly, mica-like crystals which were somewhat

[2] Hann, R., and Sando, C. E., Scyllitol from flowering dogwood (*Cornus florida*), *J. Biol. Chem.*, 1926, lxviii, 399.

smaller than those of the acetyl derivative of inositol from blackberry leaves. The compound melted sharply at 214.5–215°. The melting point leaves no doubt as to the identity of the compound with hexacetyl inositol. Analysis of the substance, dried at 115–120°, gave the following results.

0.0991 gm. gave 0.1821 gm. CO_2 and 0.0502 gm. H_2O.
Found. C 50.11, H 5.68.
Hexacetyl inositol, $C_6H_6O_6(CO \cdot CH_3)_6$, requires: C 49.98, H 5.60

SUMMARY.

Inositol, $C_6H_6(OH)_6$, has been isolated from the leaves of tall blackberry (*Rubus argutus* Link) and from the flowers and bracts of dogwood (*Cornus florida*). It was identified in each case by analysis of the purified substance and of its prepared acetyl derivative.

THE COLORING MATTER, QUERCIMERITRIN, FROM THE DOUBLE CHRYSANTHEMUM-FLOWERED SUNFLOWER (HELIANTHUS ANNUUS).

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In a paper[1] dealing with anthocyanin formation in *Helianthus annuus* it was pointed out that the yellow flavonol glucoside, quercimeritrin, had been isolated from the corollas of the double chrysanthemum-flowered sunflower. At that time no attempt was made to present in detail the method of isolation or to furnish data confirming the exact composition of the glucoside. These details are supplied in the present paper which embodies the results of a thorough investigation of the pigment.

EXPERIMENTAL.

Preparation of the Coloring Matter.—The material employed in this investigation consisted of the ligulate corollas of the so called double chrysanthemum-flowered sunflower. In all, 10,000 fully opened heads were collected from plants grown on the experimental farm at Arlington, Virginia. Of these, 3500 heads were used for the present investigation. Each day, during the flowering season, immediately after the heads were picked the corollas were carefully removed and dried in thin layers over steam pipes at 60°. From the 3500 heads, 85.5 kilos of fresh corollas, yielding 13.8 kilos of dried material, were obtained.

The dried corollas were coarsely ground in a drug mill and then extracted in 1 kilo lots by continuous percolation[2] with ether.

[1] Sando, C. E., Anthocyanin formation in *Helianthus annuus*, *J. Biol. Chem.*, 1925, lxiv, 71.

[2] Sando, C. E., Continuous extraction apparatus for large quantities of plant materials, *Ind. and Eng. Chem.*, 1924, xi, 1125.

This extraction removed materials which amounted to approximately 11 per cent of the total dry weight and consisted largely of oily substances and orange plastid pigments. A continuation of the extraction with hot redistilled 95 per cent alcohol removed practically all of the flavonol coloring matter as ascertained by tests. The filtered alcoholic solution was evaporated to a small volume and freed from alcohol by vacuum distillation. The dark colored syrup which remained in the flask was then boiled with a large volume of water. This mixture was filtered through talc on a Büchner funnel to remove insoluble viscous impurities. A saturated solution of neutral lead acetate was added to precipitate the coloring matter. This precipitate was collected on a funnel by suction, washed with hot water, and decomposed with hydrogen sulfide by suspension in hot alcohol. The liquid, after filtration from lead sulfide, was evaporated to dryness and the residue boiled with water which dissolved the crude pigment glucoside, but left suspended in the solution a dark colored, oily, viscous substance. This was difficult to remove by ordinary filtration, but its partial separation was effected by filtering the hot mixture with the aid of suction through thin layers of talc. The filtration proceeded slowly even by this method, so that it was frequently necessary to change the talc. A quantity of this oily impurity separated with the coloring matter when a portion of the clear filtrate was allowed to cool, consequently the original hot solution had to be diluted with 2 or 3 volumes of water before the solid coloring matter could be obtained in a fair state of purity. By this method, most of the remaining impurity was retained in solution without appreciably interfering with the separation of the pigment glucoside. Further purification was accomplished by successive crystallizations from boiling water, hot dilute pyridine, and again from boiling water. After this systematic and prolonged treatment there still remained a slight trace of impurity which was entirely eliminated by boiling the solid pigment with a quantity of water insufficient for solution, filtering, and then recrystallizing the insoluble residue finally from boiling water.

The total quantity of air-dried coloring matter procured by the process described above was 20.5 gm. The percentage yield calculated on the basis of material dried at 60° is, therefore, 0.148. A special extraction carried out for the purpose of deter-

mining the yield gave a higher percentage. In this determination, 1125 gm. of material, dried at 60°, yielded 3 gm. of pure, air-dried pigment, or 0.266 per cent.

Properties of the Glucoside.—The flavonol glucoside, in its final state of purity, forms a mass of glistening "reed-yellow",[3] elbow-twin, wedge-shaped crystals which are often mixed with clusters of smaller crystals as shown in Fig. 1. The substance is only slightly soluble in cold water, but more so in boiling water from which it is easily crystallized. It is readily soluble in alcohol and

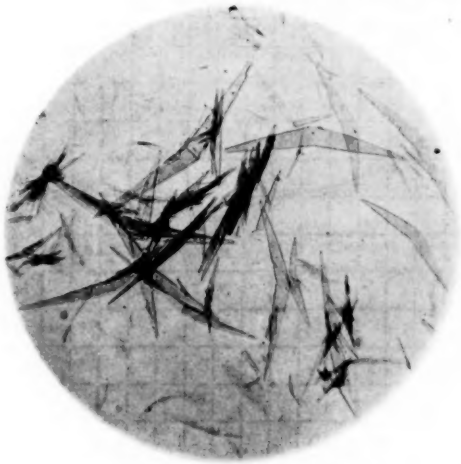

FIG. 1. Sunflower glucoside, crystallized from hot water by dissolving 0.17 gm. in 30 cc. of water (× 80).

practically insoluble in petroleum ether and ether. It dissolves in alkaline solutions with an intense yellow color and is precipitated from its neutral solutions by lead acetate as a red salt. An alcoholic solution of the pigment gives with a few drops of ferric chloride solution an olive-green color. A trace of the substance, contained in an aqueous solution (*e.g.*, the filtrate after crystallization) gives a clear pale rose-red anthocyanin color when reduced with magnesium ribbon and hydrochloric acid.

[3] Ridgeway, R., Color standards and nomenclature, Washington, 1912, plate xxx.

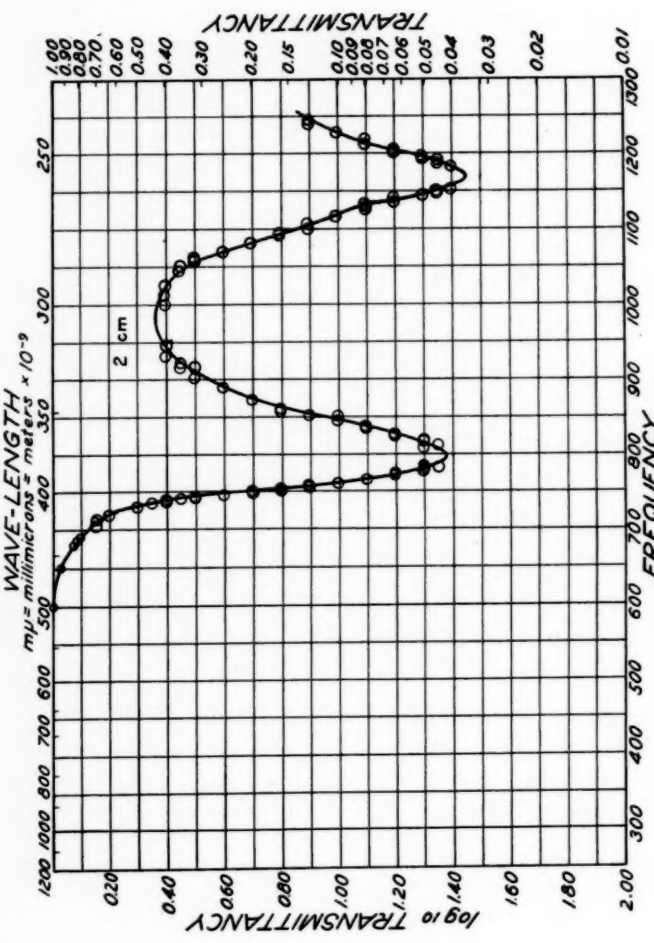

Fig. 2. The spectral transmittancy, throughout the visible and ultra-violet regions of the spectrum, of the sunflower quercimeritrin in alcoholic solution, using a 2 cm. thickness. Small circles indicate measurements by visual method, large circles represent photographic measurements.

The spectral transmittancy of the sunflower pigment is shown in Fig. 2. A solution in absolute alcohol of the pure, anhydrous substance, containing 1.392 cg. per liter or M/300,000, was used for this work. The curve was kindly prepared at the United States Bureau of Standards by Mr. H. J. McNicholas, who made the measurements in both the visible and ultra-violet portions of the spectrum.

I am also indebted to Dr. Edgar T. Wherry, of the United States Bureau of Chemistry, who has undertaken the examination of the pigment under the petrographic microscope. His results are as follows: In ordinary light, as crushed for examination by the immersion method, the substance is seen to consist of thin plates which are more or less rectangular in general outline. The color is pale yellow, and when one Nicol prism is inserted faint pleochroism is shown, the direction of index α being very pale yellow, γ pale yellow. The refractive indices are, $\alpha = 1.495 \pm 0.002$; β lies perpendicular to the plates and is not definitely determinable; $\gamma = 1.720 \pm 0.010$. Double refraction is thus 0.225, or extremely strong, this being confirmed by the bright polarization colors of the second and third orders shown on even the thinnest of plates, when examined between crossed nicols. The extinction is parallel to occasional edges, but oblique to and at varying angles to many of them. The system may thus be monoclinic, or more probably triclinic.

The pure pigment melts at 247–249°. The melting point together with other properties, especially the crystal form, indicates that the sunflower glucoside is identical with quercimeritrin which has been reported from the cotton plant, *Gossypium herbaceum*[4] and *Gossypium hirsutum*,[5] and from the bark of a species of *Prunus*.[6] A microphotograph of quercimeritrin from cotton flowers, generously supplied by Prof. A. G. Perkin, is shown for comparison in Fig. 3. Although, in the literature,[4] the melting

[4] Perkin, A. G., The colouring matter of cotton flowers, *Gossypium herbaceum*, Part II, *J. Chem. Soc.*, 1909, xcv, 2181.

[5] Viehoever, A., Chernoff, L. H., and Johns, C. O., Chemistry of the cotton plant, with special reference to Upland cotton, *J. Agric. Research*, 1918, xiii, 345.

[6] Finnemore, H., Chemical examination of a species of *Prunus*, *Pharmacol, J. and Pharmacist*, 1910, lxxxv, 604.

point of quercimeritrin is reported as 247–249° Perkin's sample, after crystallization from water, melted at 245–247°. This material, mixed in an agate mortar with an equal quantity of the sunflower pigment, did not show the depression in the melting point which would be expected if these two substances were different. The melting point of the mixture was 246–247°.

Complete identification of the sunflower coloring matter as quercimeritrin was accomplished by a thorough chemical examination which is reported below.

FIG. 3. Quercimeritrin from *Gossypium* crystallized from hot water by dissolving 0.17 gm. in 30 cc. of water (× 80).

Water of Crystallization.—The sunflower glucoside, when deposited from aqueous solution, contains three molecules of water of crystallization. This is slowly given off at 100°, but more quickly at 110–115°. The substance, which had been exposed to room air for several weeks, was heated at 110–115° until constant in weight in order to determine the loss of water.

1.2607 gm. lost 0.1300 gm. H_2O.
Found. H_2O 10.31.
Quercimeritrin, $C_{21}H_{20}O_{12}\cdot 3H_2O$, requires: H_2O 10.43.

Combustion of Anhydrous Glucoside.—The glucoside was dried to constant weight at 110–115° and analyzed with the following results.

0.1400 gm. gave 0.2790 gm. CO_2 and 0.0534 gm. H_2O.
0.1255 " " 0.2500 " " " 0.0478 " "
Found. C 54.35, 54.32; H 4.28, 4.27.
Quercimeritrin, $C_{21}H_{20}O_{12}$, requires: C 54.29, H 4.34.

Hydrolysis of Anhydrous Glucoside.—Quercimeritrin upon hydrolysis is resolved into glucose and quercetin in molecular proportions according to the equation:

$$C_{21}H_{20}O_{12} + H_2O = C_{15}H_{10}O_7 + C_6H_{12}O_6.$$

A sample of sunflower glucoside also gave glucose and quercetin upon hydrolysis. For the quantitative determination of the hydrolytic product, quercetin, the anhydrous glucoside was dissolved in 100 cc. of water and, after the addition of 4 cc. of concentrated sulfuric acid, boiled for 3 hours. After standing 24 hours in an ice box, the deposit of quercetin was collected in a weighed Gooch crucible and dried at 130°.

0.5345 gm. gave 0.3460 gm. quercetin.
0.4906 " " 0.3179 " "
Found. Quercetin 64.73, 64.80.
Quercimeritrin requires: quercetin, $C_{15}H_{10}O_7$, 65.08.

Combustion of Quercetin.—The quercetin obtained by the hydrolysis of the sunflower glucoside was converted into its acetyl derivative by boiling with acetic anhydride and anhydrous sodium acetate. The acetyl compound was recrystallized several times from 70 per cent alcohol and dried. It formed a felty mass of glistening, colorless needles melting at 194–196°. Hydrolysis of this derivative was effected by solution in glacial acetic acid and boiling after the addition of 3 drops of concentrated sulfuric acid. The liberated quercetin slowly separated upon the addition to the mixture of an excess of water. It was collected and recrystallized from dilute alcohol. After drying at 130°, it was analyzed with the following results.

0.1816 gm. gave 0.3973 gm. CO_2 and 0.0522 gm. H_2O.
0.1170 " " 0.2554 " " " 0.0349 " "
Found. C 59.66, 59.53, H 3.22, 3.34.
Quercetin, $C_{15}H_{10}O_7$, requires: C 59.59, H 3.93.

Combustion of Pentacetyl Quercetin.—This derivative was prepared as described above. It was dried at 160° and analyzed.

0.1201 gm. gave 0.2583 gm. CO_2 and 0.0422 gm. H_2O.
0.1308 " " 0.2819 " " " 0.0448 " "
Found. C 58.77, 58.65; H 3.84, 3.94.
Pentacetyl quercetin, $C_{18}H_5O_7(CO \cdot CH_3)_5$, requires: C 58.57, H 3.93.

Hydrolysis of Pentacetyl Quercetin.—Purified pentacetyl quercetin was dried at 160°, then hydrolyzed in glacial acetic acid to which a few drops of concentrated sulfuric acid had been added. The liberated quercetin was obtained by adding water and allowing the mixture to stand in an ice box for 24 hours. It was then collected in a weighed Gooch crucible, washed with water, and dried at 130°.

0.6045 gm. gave 0.3548 gm. quercetin.
0.5233 " " 0.3067 " "
Found. Quercetin 58.71, 58.61.
Pentacetyl quercetin requires: quercetin 58.98.

SUMMARY.

A quercetin monoglucoside, $C_{21}H_{20}O_{12} \cdot 3H_2O$, has been isolated to the extent of 0.266 per cent from the dried ligulate corollas of the so called chrysanthemum-flowered sunflower (*Helianthus annuus*). As a result of a thorough study, including a chemical examination, it is concluded that the glucoside is identical with quercimeritrin which has been isolated from the cotton plant, *Gossypium herbaceum* and *Gossypium hirsutum* and from the bark of a species of *Prunus*.

ON THE CONFIGURATIONAL RELATIONSHIP OF β-HYDROXYBUTYRIC ACID AND PROPYLENE GLYCOL.

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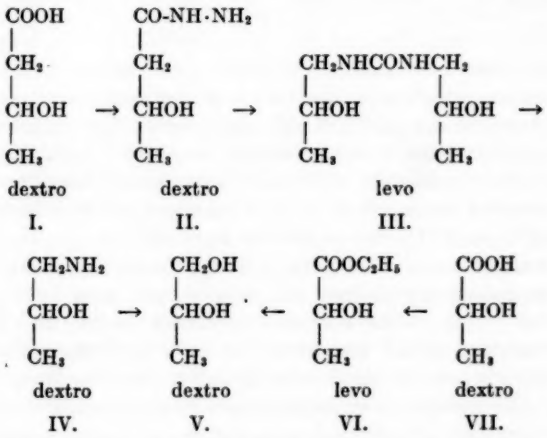
The configurational relationship of β-hydroxybutyric and lactic acids was recently formulated by Levene and Haller[1] in the sense that the two configurationally related substances rotate in the same direction.

The set of reactions which led to this conclusion is the following.



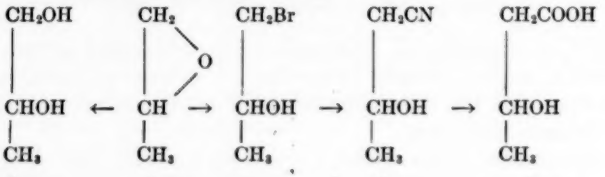
Karrer and Klarer[2] independently and simultaneously carried out the conversion of dextro-β-hydroxybutyric acid into dextropropylene glycol by the Hofmann method.

[1] Levene, P. A., and Haller, H. L., *J. Biol. Chem.*, 1925, lxv, 49; 1926, lxvii, 329.

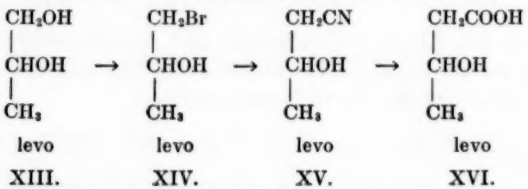
[2] Karrer, P., and Klarer, W., *Helv. Chim. Acta*, 1925, viii, 393.

From the above figures it is seen that both dextro-β-hydroxybutyric and dextro-lactic acids are converted into dextro-propylene glycol. Hence, the configurational relationship of dextro-β-hydroxybutyric acid with dextro-propylene glycol is the essential part of the argument in favor of the relationship given at the beginning of this paper. However, Abderhalden and Eichwald,[3] at an earlier date, came to the conclusion that dextro-propylene glycol is configurationally related to levo-β-hydroxybutyric acid.

The set of reactions which led Abderhalden and Eichwald to their conclusions was the following.



It is seen that their argument is based on two assumptions: First, that dextro-propylene oxide (IX) is configurationally related to levo-propylene bromohydrin (X); and second, that dextro-propylene oxide is related to dextro-propylene glycol (VIII). *A priori*, there exists a possibility of Walden inversion in either one of the two reactions inasmuch as in both reactions, namely in the one from (IX) to (VIII) and in the one from (IX) to (X), the asymmetric carbon atom is involved. It was therefore desired to convert propylene glycol into β-hydroxybutyric acid by a set of reactions which would not involve carbon atom (2). Hence, levo-propylene glycol was converted into the bromohydrin, the bromohydrin into the nitrile, and the latter into β-hydroxybutyric acid. The results of our experiments were as follows:



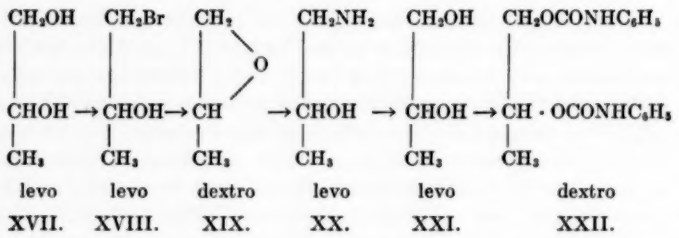
[3] Abderhalden, E., and Eichwald, E., *Ber. chem. Ges.*, 1918, li, 1312.

Thus it is seen that reactions (XIV) to (XVI) proceeded in our set of reactions in the same way as (X) to (XII) of Abderhalden and Eichwald. On the other hand, the relationship between (XIV) and (XIII) is opposite to that between (X) and (VIII).

The question arises as to which of the two sets of reactions leads to the correct conclusion. It is significant in this connection that Abderhalden and Eichwald give no reason for the assumption of the relationship between (VIII) and (IX). In fact, they definitely state that dextro-propylene oxide leads to either dextro- or levo-propylene glycol and that the course of the reactions could not be controlled by them. Thus, the conclusions of Abderhalden and Eichwald fall on the basis of their own evidence. On the other hand, the set of reactions from (XIII) to (XVI) is more likely to proceed without Walden inversion. Hence, the inference based on them seems quite warranted. The inference gains in weight because it is supported by the evidence furnished earlier by Levene and Haller.

In the argument thus far presented, no experimental proof has been given for the structure of the bromohydrin as given in figure (XIV). The proof, however, is desired in view of the fact that this compound had not been previously prepared by exactly the same method as described in this paper. In order to prove that the substance actually was 1-bromo-2-hydroxypropane, our propylene bromohydrin was oxidized with chromic acid. The reaction product was found to be bromoacetone, whereas 1-hydroxy-2-bromopropane on oxidation yields bromopropionic acid. The bromoacetone was identified by its conversion into acetol. The acetol in its turn was converted into its *p*-nitrophenylhydrazone, or into its *p*-nitrophenylosazone.

Occasion was also taken to verify our earlier conclusion in regard to the configurational relationship between propylene glycol and 1-amino-2-hydroxypropane. This relationship is the decisive one in correlating β-hydroxybutyric acid with propylene glycol. In the experiments of Karrer and Klarer as well as in those of Levene and Haller on the degradation of β-hydroxybutyric acid, no crystalline derivatives of the glycol were prepared. We therefore prepared 1-amino-2-hydroxypropane from the optically active propylene oxide. The base was then converted into the glycol and the glycol into the di- (phenylurethane).



For comparison the urethane was also prepared directly from levo-propylene glycol and the two urethanes were found to be identical.

CONCLUSIONS.

1. Levo-propylene glycol was converted into 1-bromo-2-hydroxypropane.

2. The above bromohydrin was converted into levo-β-hydroxybutyric acid.

3. Levo-1-amino-2-hydroxypropane was converted into levo-propylene glycol.

4. These observations confirm the conclusions of Karrer and Klarer and of Levene and Haller regarding the configurational relationship of β-hydroxybutyric acid and propylene glycol.

5. They also confirm the conclusion of Levene and Haller regarding the configurational relationship of β-hydroxybutyric and lactic acids.

EXPERIMENTAL.

Preparation of Propylene Bromohydrin.—Into 20 gm. of optically active propylene glycol[4] ($[\alpha]_D^{20} = -14.57°$) 22 gm. of dry hydrogen bromide (1 equivalent) were passed at 0°C. This solution was heated in a water bath for 1 hour. To the reaction product chloroform and ice water were added and the mixture was made neutral to Congo red. The chloroform was separated and the extraction with chloroform was repeated twice. The combined chloroform extracts were dried over sodium sulfate. The chloroform was removed by distillation and the remaining propylene bromohydrin was distilled at 45°C. at 12 mm. or at

[4] Farber, E., Nord, F. F., and Neuberg, C., *Biochem. Z.*, 1920, cxii, 313.

144° at 760 mm. The yield of propylene bromohydrin was 24 gm. The substance analyzed as follows:

0.1290 gm. substance: 0.1752 gm. AgBr.
C_3H_7OBr. Calculated. Br 57.55.
Found. " 57.80.

It gave the following rotation without solvent:

$$[\alpha]_D^{20} = \frac{-4.61°}{1 \times 1.5988} = -2.87°.$$

When hydrogen bromide is passed into propylene glycol at 100°, the reaction is completed in less than 2 hours. The product, however, is not quite so pure as the one obtained by the first process. The unchanged propylene glycol can be readily recovered. For this purpose, the aqueous portion remaining after chloroform extraction is concentrated under diminished pressure and at room temperature. The residue is extracted with alcohol and the extract is fractionated.

Oxidation of Propylene Bromohydrin.—20 gm. of potassium bichromate were dissolved in 100 cc. of water to which 17.0 gm. of sulfuric acid had been added. The solution was warmed to 30°C. and to it 15 gm. of the bromohydrin were added. The temperature of the solution rose spontaneously to 52°C. and was maintained at 55°C. for ½ hour. The reaction product was then extracted with ether, the ethereal solution was dried over sodium sulfate, freed from ether, and then fractionally distilled under diminished pressure.

The fraction distilling from 38–45°C. at 12 mm. pressure was collected and analyzed.

0.1510 gm. substance: 0.2080 gm. AgBr.
C_3H_5OBr. Calculated. Br 58.40.
Found. " 58.62.

Only a small fraction remained in the distilling flask. It analyzed for unchanged propylene bromohydrin, thus showing that bromopropionic acid was absent in the products of oxidation.

Conversion of Bromoacetone into Acetol.—The bromoacetone described above was converted into acetol by means of potassium formate in methyl alcohol in the usual way. It was found

convenient to identify the substance by its nitrophenylhydrazine derivatives, since both the hydrazone and the osazone were readily obtained.

The hydrazone was obtained in the following manner. To a solution of acetol in methyl alcohol 1 equivalent of *p*-nitrophenylhydrazine in acetic acid solution was added. To this solution water was added to incipient opalescence. The hydrazone began to crystallize immediately in the form of light yellow irregular platelets. The hydrazone was filtered, washed with alcohol, and analyzed without further purification. The substance melted at 173°C. and analyzed as follows:

0.0540 gm. substance: 10.00 cc. N, p = 754.3 mm., t = 17°C.
$C_9H_{11}O_3N_3$. Calculated. N 20.09.
Found. " 21.62.

In order to prepare the osazone 3 equivalents of *p*-nitrophenylhydrazine were added to an alcoholic solution of acetol. Then water was added to opalescence and the solution was boiled over a free flame until all the osazone settled out in long dark red needles. The osazone can be recrystallized either from dilute methyl alcohol, or better, from a large volume of glacial acetic acid. Before drying, the osazone should be washed with alcohol and ether and then dried under reduced pressure at the temperature of toluene vapor. The substance decomposes with effervescence at 291°. The analysis of the substance was as follows:

0.0850 gm. substance: 0.1635 gm. CO_2 and 0.0346 gm. H_2O.
0.0560 " " : 11.80 cc. N, p = 754.6 mm., t = 20°C.
$C_{15}H_{14}N_6O_4$. Calculated. C 52.61, H 4.12, N 24.79.
Found. " 52.45, " 4.55, " 24.38.

For comparison, acetol was prepared from bromoacetone derived from acetone. From this acetol, a *p*-nitrophenylosazone was obtained in the same manner as above. It had the same appearance, decomposed at 293°, and analyzed as follows:

0.1006 gm. substance: 0.1942 gm. CO_2 and 0.0398 gm. H_2O.
0.0526 " " : 11.20 cc. N, p = 754.2 mm., t = 23°C.
$C_{15}H_{14}N_6O_4$. Calculated. C 52.61, H 4.12, N 24.79.
Found. " 52.64, " 4.42, " 24.36.

β-Hydroxybutyronitrile.—Equal parts of pulverized potassium cyanide and optically active propylene bromohydrin were taken up in five parts of dry methyl alcohol and refluxed overnight. After cooling and filtering, the methyl alcohol was removed by distillation under reduced pressure and the residue was extracted with ether. The ethereal solution was dried and the ether was then evaporated. The β-hydroxybutyronitrile distilled at 99–100°C. at 12 mm. The specific rotation in water was:

$$[\alpha]_D^{25} = \frac{-0.48^\circ \times 100}{1 \times 4.76} = -10.08^\circ.$$

The substance analyzed as follows:

0.1022 gm. substance required for neutralization 12.00 cc. 0.1 N HCl.
C_4H_7ON. Calculated. N 16.47.
Found. " 16.43.

β-Hydroxybutyric Acid.—5 gm. of β-hydroxybutyronitrile were taken up in 40 cc. of concentrated hydrochloric acid and refluxed for 2 hours. The solution was then evaporated under reduced pressure, water being added several times during the distillation in order to remove the hydrochloric acid. The remaining hydrochloric acid was removed by silver sulfate and the filtrate from the silver chloride was extracted for 24 hours with ether in a continuous extractor. The ether solution was then dried over sodium sulfate and concentrated. The residue was dissolved in absolute alcohol. An aliquot part of the solution was titrated with 0.1 N sodium hydroxide and the calculated amount of 1 N sodium hydroxide was added to the main part of the solution which was taken up in a double-neck flask provided with a separatory funnel containing absolute alcohol, and was distilled under diminished pressure. When nearly all the water had been distilled over, alcohol was added and the distillation continued. The addition of alcohol was repeated several times. Towards the end of the operation, the residue consisted of a crystalline deposit of microscopic needles. The substance analyzed as follows:

0.1028 gm. substance: 0.0572 gm. Na_2SO_4.
$C_4H_7O_3Na$. Calculated. Na 18.25.
Found. " 18.01.

It gave the following rotation in water.

$$[\alpha]_D^{20} = \frac{-0.89^\circ \times 100}{1 \times 8} = -11.12^\circ.$$

Dextro-Propylene Oxide.—A double-neck distilling flask containing 19 gm. of sodium hydroxide and 40 cc. of water was immersed in a water bath. The flask was connected with a condenser through which water of 0°C. was circulating. The temperature of the water bath was then raised to 40°C. When this temperature was reached, the bromohydrin was added to the akaline solution and the temperature of the water bath was quickly raised to 60°. The flask was vigorously shaken while the propylene oxide was distilling. The substance had the following specific rotation without solvent.

$$[\alpha]_D^{20} = \frac{+9.25^\circ}{1 \times 0.8412} = +11.57^\circ.$$

Conversion of Dextro-Propylene Oxide into Levo-1-Amino-2-Hydroxypropane.—14 gm. of the propylene oxide described above were taken up in 500 cc. of concentrated aqueous ammonia at 0°C. and allowed to stand in the refrigerator overnight and an additional 3 hours at room temperature. The solution was then concentrated under reduced pressure at room temperature until the odor of ammonia disappeared. The residue was distilled at 2 mm. pressure and the distillate was received in a flask which was cooled in a mixture of solid carbon dioxide and ether. After a small part of the contents had been distilled, the distillate began to crystallize in the side tube of the distilling flask. The distillation was then interrupted and both the residue and the distillate were taken up in alcohol containing 20 per cent of hydrogen chloride. The solution was cooled and dry ether was gradually added to it. The hydrochloride of the base then crystallized in heavy prisms. It analyzed as follows:

0.1000 gm. substance required 8.90 cc. 0.1 N $AgNO_3$.
0.0200 " " : 4.55 cc. N, p. = 751.3 mm., t = 22°C.
$C_3H_9ON \cdot HCl$. Calculated. N 12.56, Cl 31.83.
Found. " 12.60, " 31.59.

The rotation of the substance in water was:

$$[\alpha]_D^{20} = \frac{-1.34^\circ \times 100}{1 \times 4} = -33.5^\circ.$$

Conversion of the Amine into Propylene Glycol.—A solution at 0°C. of 10 gm. of potassium nitrite in 40 cc. of water was slowly added to a cold solution of 10 gm. of the above hydrochloride in 100 cc. of water, the mixture being agitated continuously during the operation. The solution was then allowed to stand in a refrigerator overnight. The following morning, 6 gm. of potassium nitrite and an equivalent of hydrochloric acid were added. At the end of the operation, the solution reacted neutral to Congo red and acid to litmus. It was then concentrated under reduced pressure, the temperature of the water bath being maintained at 35°C. The residue, consisting of propylene glycol and a considerable sediment of mineral salts, was extracted with a mixture of two parts of alcohol and one part of ether. The extract was freed from alcohol and ether and the residue was distilled. The fraction distilling between 85–91° at 12 mm. was collected. The rotation of the glycol in approximately 10 per cent solution in water was $[\alpha]_D^{20} = -9.8°$.

Conversion of Levo-Propylene Glycol into Dextro-Propylene Glycol Di- (Phenylurethane).—0.5 gm. of the glycol and 2 gm. of phenylisocyanate were placed in a small test-tube provided with a reflux condenser carrying a calcium chloride tube at the upper end. The tube was heated in a metal bath 1½ hours at 100°C. The substance then crystallized.

For analysis the substance was recrystallized from dilute alcohol. It had a melting point of 143–144° and analyzed as follows:

0.0500 gm. substance required 3.20 cc. 0.1 N HCl.
$C_{17}H_{18}N_2O_4$. Calculated. N 8.91.
Found. " 8.96.

The rotation of the substance in absolute alcohol was:

$$[\alpha]_D^{20} = \frac{+0.38° \times 100}{1 \times 4} = +9.5°.$$

Conversion of the Fermentation Levo-Propylene Glycol into Propylene Glycol Di- (Phenylurethane).—For comparison, the urethane was prepared also from levo-propylene glycol obtained by the fermentation of acetol.

To 2.5 gm. of active propylene glycol ($[\alpha]_D^{20} = -15.37°$) were added 8 gm. of phenylisocyanate. This mixture was heated with frequent shaking on the water bath until it became solid (about 1 hour). The solid mass was treated in a mortar with dry ether, filtered, and crystallized from dilute alcohol. It yielded 5.5 gm. of urethane. The substance had a m.p. of 146–147°, and analyzed as follows:

0.1000 gm. required for neutralization 6.25 cc. 0.1 N acid.
$C_{17}H_{18}N_2O_4$. Calculated. N 8.91.
Found. " 8.75.

The rotation of the substance in absolute alcohol was as follows:

$$[\alpha]_D^{20} = \frac{+0.40° \times 100}{1 \times 3} = +13.3°.$$

ROTATORY AND REDUCING VALUES OF GLUCOSE AS INFLUENCED BY THE ADDITION OF MUSCLE TISSUE AND INSULIN IN VITRO.

By JOHN R. PAUL.*

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In a series of experiments Lundsgaard and Holbøll (1, 2) have recently studied the effect of insulin upon the sugar molecule *in vitro*. Their results seem to add support to the theory that insulin may exert a direct influence upon the sugar molecule. The experiments consisted of a careful study of a series of glucose solutions to which muscle tissue and insulin had been added. The solutions were first rotated in the water bath at 37° for several hours, then portions were dialyzed through collodion sacs, and in the dialysates thus obtained the ratio between the rotatory and reducing power of the dialysate was tested. They found that within 2 hours the former was diminished as compared to the latter with a subsequent approach towards the normal ratio for α,β-glucose at the end of 24 hours. In their first publication (1) this phenomenon was interpreted as showing, either that a disturbance in the normal equilibrium of α,β-glucose had taken place or that possibly γ-glucose, which has a lower specific rotation than that of equilibrated α,β-glucose, had been formed. In a later publication (2) the original observations were amplified by a comparative study of the mutarotation of β-glucose and the glucose transformed by insulin and muscle tissue. They concluded that the diminution in rotatory power of a glucose solution caused by insulin and muscle tissue could not be due to an increase of β-glucose but must be caused by the presence

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of a new form of glucose with a low rotatory power for which the term "new-glucose" was proposed. The report is of considerable interest and the phenomenon described would seem to merit further careful study as the new-glucose theory evidently is of fundamental importance in our conception of carbohydrate metabolism.

Barbour (3) has recently repeated the original experiments of Lundsgaard and Holbøll and has been unable to confirm the findings just described. He did not detect any change in rotatory or reducing power after 2 hours in solutions of glucose to which muscle tissue and insulin had been added.

It is to be recalled that the same general hypothesis, that a diminution in the optical activity of α,β-glucose may represent a step in the preparation of glucose for utilization by the body, has been suggested by the work of Admont Clark (4), and again by Winter and Smith (5). The conclusions of the latter investigators have however, not been entirely accepted by Hewitt (6), Eadie (7), Van Creveld (8), Visscher (9), and Denis and Hume (10).

I have recently attempted to duplicate the original experiments of Lundsgaard and Holbøll by a study of the rotatory and reducing power of both dialysates and ultrafiltrates from solutions of glucose to which muscle tissue and insulin had been added. By the use of ultrafiltration in this work one can study the rotatory and reducing power of glucose solutions in which the concentration more nearly approaches that of the original solution, as well as the ratio between rotatory and reducing power.

Methods.

Merck's pure dextrose was used in all of the experiments. The insulin employed was the commercial product of Eli Lilly and Company. The muscle tissue was obtained from freshly killed guinea pigs.

The collodion sacs were prepared from a 7 per cent solution of Du Pont's parlodion dissolved in a solution of 75 per cent by volume of ether and 25 per cent of absolute alcohol. It is evident that in making sacs for this purpose the degree of permeability is a factor of considerable importance. In the preliminary experimentation on the preparation of suitable collodion mem-

branes the principles emphasized by Eggerth (11) were followed. The eventual standard which was adopted was that the sacs should be absolutely impermeable to solutions of hemoglobin but quite permeable to glucose. In making a sac the inside of a test-tube was coated with collodion, drained, and allowed to dry for about 4 or 5 minutes until the collodion was quite firm. The tube was then filled to the brim with 70 per cent alcohol and allowed to stand for 20 minutes. Distilled water was then substituted for the alcohol for a period of 1 hour. At the end of this time the sac was usually found to have contracted slightly from the tube wall and could be drawn out with relative ease. The sacs were then tested for leaks and hemoglobin impermeability by filling them with a solution of hemoglobin and subjecting them to a negative pressure of 200 mm. of mercury. The selected ones were kept in water to which a few crystals of thymol had been added and were used repeatedly over a period of about 3 to 4 weeks. The method of ultrafiltration was that described by Marshall and Vickers (12). A negative pressure of 150 to 200 mm. of mercury was employed.

Polariscopic readings were made with a Reichert instrument, using a 189 mm. tube, with a Mazda lamp and an appropriate dichromate solution as the light source. A specific rotation of $+52.8°$ was used throughout the calculations. The readings were made at constant temperature and in every instance the average of a series of six individual readings in which the variation did not exceed $0.04°$ was taken. A series of averaged readings expressed in terms of glucose per cent, made in this manner from a 1 per cent glucose solution, is shown in Table I. It will be noted that the maximum error is -0.018 gm. or about 2 per cent.

In selecting a suitable reducing method for the determination of glucose in the ultrafiltrates and dialysates a certain amount of preliminary study was required. Three reducing methods were tried; namely, that of Shaffer and Hartmann (13), Folin and Wu (14), and Hagedorn and Jensen (15). The first of these three methods (Shaffer and Hartmann) proved to be the most satisfactory in these experiments for the determination of glucose in a concentration of 1 per cent. The last method (Hagedorn and Jensen) was that used by Lundsgaard and Holbøll.

In Table I a series of individual reducing determinations by each method is recorded together with the deviation from the mean determination. The use of triplicate titrations in the first and third methods, however, materially reduced the error to approximately 1 per cent.

Hydrogen ion determinations were made by colorimetric comparison with standard indicators and buffer solutions.

A preliminary series of experiments was run on ultrafiltrates

TABLE I.

Degree of Error for Individual Rotatory and Reducing Determinations by Three Different Reducing Methods.

Rotatory determinations.		Reducing determinations.					
Solution I.*		Shaffer and Hartmann. Solution II.		Folin and Wu. Solution III.		Hagedorn and Jensen. Solution IV.	
Glucose concentration.	Deviation from mean.	Glucose concentration.	Deviation from mean.	Glucose concentration.	Deviation from mean.	Glucose concentration.	Deviation from mean.
per cent		*per cent*		*per cent*		*per cent*	
1.001	+0.011	1.018	+0.016	0.960	−0.010	0.990	−0.007
0.972	−0.018	1.003	+0.001	0.970	0.000	0.990	−0.007
1.002	+0.012	0.994	−0.008	0.970	0.000	1.000	+0.003
1.000	+0.010	0.985	−0.017	0.960	−0.010	1.020	+0.023
0.975	−0.015	1.015	+0.013	0.980	+0.010	0.990	−0.007
0.992	+0.002	1.000	−0.002	0.980	+0.010	1.000	+0.003
Average. 0.990		1.002		0.970		0.997	

* Each method was run upon a different glucose solution supposedly of a concentration of 1 per cent, but all the determinations by any one method were on the same solution and were performed within 6 hours.

of plain Locke's solution to which muscle tissue had been added. The presence of optically active or reducing substances was not detected in these solutions.

EXPERIMENTAL.

On the day previous to each experiment 500 cc. of a solution of glucose in varying concentrations of from 1.0 to 0.3 per cent was prepared in Locke's solution and allowed to stand in the ice box overnight. In the earlier series 20 cc. of phosphate buffer

solution (M/15 Na_2HPO_4 and KH_2PO_4 in appropriate concentration to yield a pH of 7.2) were added. An initial rotatory and reducing determination was run on this original solution to exclude any mutarotatory phenomena.

The original solution was divided into three flasks, A, B, and C, containing 100 cc. each, and 15 to 20 gm. of freshly minced guinea pig muscle were then added to Flasks A and B. To Flasks A and C, 20 to 60 units of insulin were added, Flask B being diluted accordingly. The final total volume in Flask A or B was about 120 cc. and in Flask C, 101 to 103 cc., depending on the amount of insulin used. This gave two controls for every experiment, one containing glucose solution and muscle tissue and the other containing glucose solution and insulin.

In the first series of experiments the three flasks were rotated in a constant temperature water bath at 37° for a period of from 3 to 4 hours and, at hourly intervals, samples were withdrawn from each flask and placed in the collodion sacs for ultrafiltration at room temperature. After 2 hours of filtration generally enough filtrate had been obtained for a rotatory and reducing determination. As a rule three or four consecutive filtrate samples were obtained for study from each flask. The experiment was limited to a study of these samples over a period of about 3 hours.

In the second series of experiments the three flasks were not rotated, but incubated at 37° for a period of from 1 to 2 hours. The contents of each flask was then divided and 50 cc. were placed in a collodion sac for ultrafiltration at room temperature, while 25 cc. were placed in another sac for dialysis against an equal volume of distilled water. A single ultrafiltrate and dialysate were obtained from each of the three flasks and the experiment was limited to a study of these single specimens. Rotatory determinations were made throughout a period of 3 to 4 hours and the specimens were then placed in the ice box overnight for a final rotatory examination on the following day. An initial and final reducing determination by the Hagedorn and Jensen method was also made on each specimen.

A frequent source of error proved to be that of bacterial contamination and growth in the ultrafiltrates which had been exposed to muscle tissue. This could be detected, however, by a

clouding of the solution which was quite noticeable in the polariscope tube, and also by the fact that both reducing and rotatory determinations showed a progressive parallel fall. The results were, of course, discarded in those experiments in which bacterial contamination was present.

Results.

The results of the two series of experiments were slightly different. In the first series seven experiments were performed in which Flask A, containing muscle tissue and insulin, Flask B, muscle tissue alone, and Flask C, insulin alone, were rotated in the water bath and consecutive filtrate samples were studied. In this series filtrates from Flasks A and B, both containing muscle tissue, showed a fairly consistent immediate loss in sugar content as estimated by the reducing determinations. This loss represented between 10 and 20 per cent of the original concentration and is graphically shown in Fig. 1 by the points grouped below lines A and B. This phenomenon was not carefully studied as it was somewhat apart from the problem under investigation. It was more marked and more extensive in the first series of experiments in which rotation of the flasks produced partial disintegration of the muscle tissue. Evidence seems to suggest, however, that this loss was largely due to a dilution of the hypertonic glucose solution by tissue fluids extracted from the added 15 to 20 gm. of muscle. The fact that the loss represented an immediate drop in sugar content rather than a progressive one and was not accompanied by the presence of bacteria in the filtrates, suggested that it probably was not due to bacterial growth. It would also seem that this loss was too precipitous and too extensive to be quite comparable to a glycolytic effect similar to that produced by the addition of red blood cells to a glucose solution. It was, of course, necessary to follow these changes in reducing values quite closely if one was to compare the reducing values of the glucose present with the rotatory values. The glucose content computed from the polariscopic readings showed a somewhat similar fall in the early part of the experiment but in general the curves computed from estimations based on the rotatory power differed somewhat from those based on reducing determinations. As a rule the rotatory values

were found to be slightly higher than the reduction values. In Fig. 2 the ratios of rotatory values to the reducing values, expressed as per cent of glucose, have been charted. It will be seen that the deviations between rotatory and reducing values

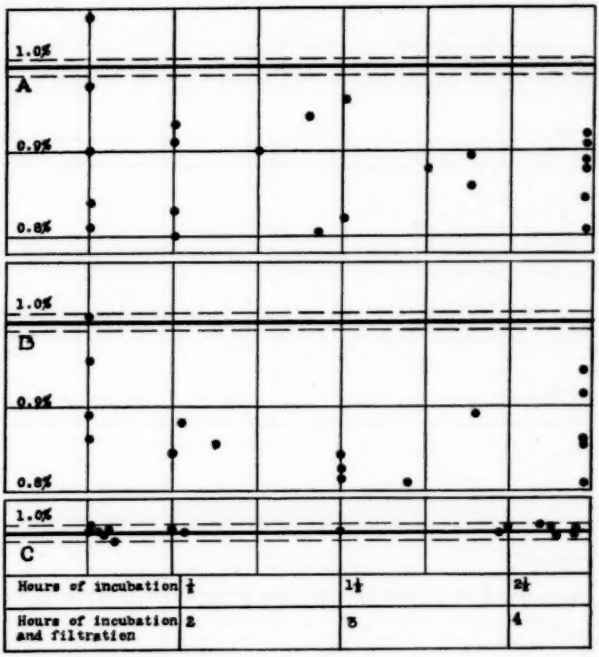

FIG. 1. Reducing values of ultrafiltrates. First series. *A*, glucose-muscle tissue with insulin. *B*, glucose-muscle tissue without insulin. *C*, control glucose solution with insulin.

The broken lines indicate the error of the analytical method. The diminution of the reducing values in *A* and *B*, from the initial concentration of 1 per cent, is presumably due largely to dilution of the glucose by the addition of muscle tissue.

are quite appreciable and they also seem to follow a definite trend consisting of a gradual increase of the former during the 3 hour period in which the filtrates were studied. In Solution A (glucose-muscle plus insulin) the deviations are divided above

and below the zero line at the 2 hour mark, but later the tendency is for these values to rise. In control Solution B (glucose-muscle tissue) the rotatory values are distinctly greater than the reduction ones throughout. Owing to these somewhat irregular differences of ratio in the control Solution B, an attempt to

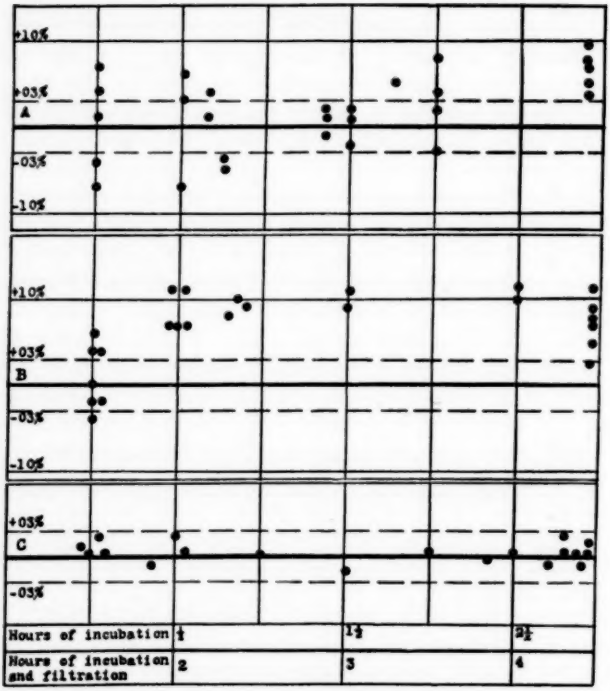

FIG. 2. Ratio $\frac{\text{rotatory value}}{\text{reducing value}}$ of ultrafiltrates. First series. The ratio values are expressed in per cent.

analyze the curve which might be drawn through the points obtained from Solution A was not considered practical. In the case of both Solutions A and B the discrepancies between rotatory and reducing values are greater than in the control Solution C (glucose and insulin), for in the latter case the values fall within the limits of the error of the methods.

Finally hydrogen ion determinations were made on a number of the filtrates from Solutions A, B, and C. As has been noted by other workers (2, 3), a fall in pH occurs in the solutions containing muscle tissue. In these experiments it reached about 6.5 to 6.6 at the end of 3 hours, although after this the values remained fairly constant. The presence of lactic acid which may have contributed towards rendering the solutions acid was repeatedly shown in these filtrates. The changes in pH were not

TABLE II.

Reducing and Rotatory Determinations in Four Experiments. Second Series.

	Filtrate from control glucose solution.	Filtrate from glucose solution plus muscle tissue.			Filtrate from glucose solution plus muscle tissue and insulin.			Filtrate from control glucose solution.
	At beginning of experiment.	2 hrs.	4 hrs.	24 hrs.	2 hrs.	4 hrs.	24 hrs.	At end of experiment.
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Reducing...	0.990	0.860		0.835	0.890		0.860	0.970
Rotatory...	0.996	0.920	0.903	0.835	0.879	0.885	0.872	0.991
Reducing...	0.980	0.950		0.950	0.910		0.920	0.980
Rotatory...	0.976	0.952	0.948	0.927	0.914		0.919	1.000
	Dialysate from control glucose solution.	Dialysate from glucose solution plus muscle tissue.			Dialysate from glucose solution plus muscle tissue and insulin.			Dialysate from control glucose solution.
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Reducing...	0.253	0.180		0.180	0.220		0.188	0.253
Rotatory...	0.267	0.150	0.140	0.160	0.172	0.161	0.185	0.249
Reducing...	0.163	0.133		0.129	0.145		0.158	0.169
Rotatory...	0.148	0.130	0.163	0.159	0.148	0.176	0.148	0.152

sufficient to account for the changes in optical activity by virtue of their effect on the specific rotation of glucose but they suggested how extensive and perhaps varied were other chemical changes that were occurring in such a composite mixture.

In the second series of experiments, single samples from Flasks A, B, and C, which had been incubated over a period of from 1 to 2 hours, were divided into two portions, one of which was subjected to ultrafiltration, while the other was dialyzed against an equal volume of distilled water. The resulting solutions were

studied over a period of 24 hours. Figures from a few of the fifteen experiments performed in this manner are given in Table II. Two experiments are shown in which the samples for analysis were obtained by ultrafiltration and two by dialysis. Slight but

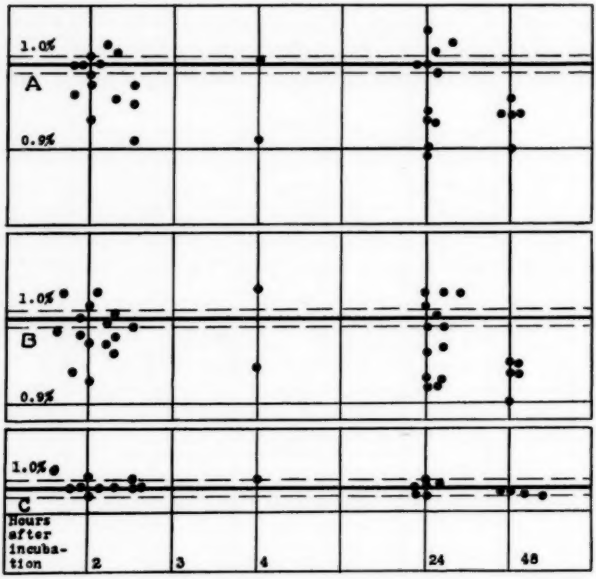

FIG. 3. Reducing value of ultrafiltrates and dialysates. Second series. Ultrafiltration or dialysis was begun after incubating the mixtures from 1 to 3 hours. The intervals between commencing ultrafiltration or dialysis and the analysis are indicated as abscissæ.

definite evidence of an actual fall in glucose concentration occurring in those solutions to which muscle tissue has been added is again noted, and is graphically shown by the reduction values charted in Fig. 3. It will be seen by comparison, however, that the degree of this loss is less than that exhibited by the first series. In general the same discrepancies of the ratio between rotatory and reducing values are again noted in the samples to which muscle tissue has been added (Fig. 4). In the case of the control solutions the points fall within the limits of error of

the methods. We were unable to detect any consistent trend in this discrepancy by which the samples to which insulin and muscle tissue had been added might be differentiated from other samples to which muscle tissue alone had been added.

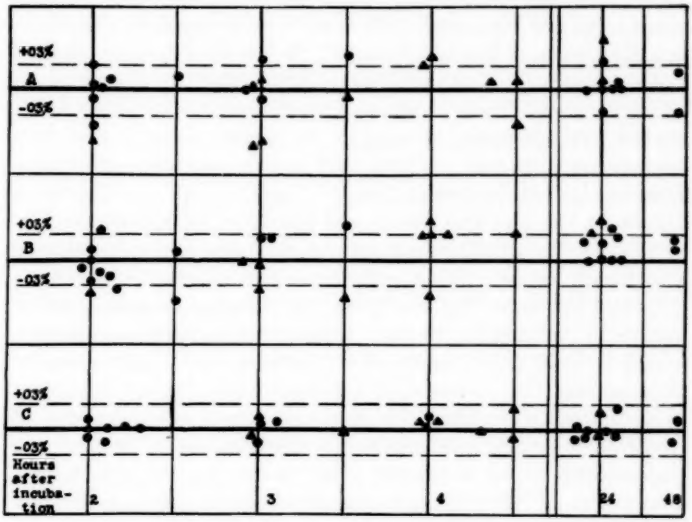

FIG. 4. Ratio $\frac{\text{rotatory value}}{\text{reducing value}}$ in ultrafiltrates and dialysates. Second series. The ratio values are expressed in per cent. The abscissæ are the same as in Fig. 3.

●, ratio from determined rotatory and reducing value.

▲, ratio from a determined rotatory value and from a reducing value interpolated between original and final reducing value on the same ultrafiltrate or dialysate.

DISCUSSION.

The reader will appreciate that the study has consisted largely of an attempt to control the factors of error which might lead to discrepancies between rotatory and reducing determinations of sugar solutions to which muscle tissue has been added. These experiments deal with such a composite mixture that theoretically the variable factors are many, particularly when one considers

that both optical activity and reducing power are far from being specific properties of glucose. For this reason, deviations in the polariscopic readings have not been expressed as representing deviations of the standard specific rotation of glucose. The discrepancies between rotatory and reduction values have not been great and have been noted with considerable irregularity throughout the course of the experiments. It has already been pointed out by others (7) that such discrepancies might be produced by the presence of traces of other optically active substances which do not have the same reducing power as glucose and which may be relatively unstable. That such other optically active substances do not represent purely hypothetical substances is shown by the fact that lactic acid has been repeatedly demonstrated in the utrafiltrates from the solutions containing glucose and muscle tissue. Other optically active substances which are hypothetical, from the standpoint of these experiments, which might be permeable to the collodion membranes employed, could include amino acids and glucuronic acid. Attempts to demonstrate the presence of optically active substances other than glucose were made by removing the sugar by means of yeast fermentation and refiltering the solution. These proved unsuccessful in our hands owing to the fact that after fermentation traces of reducing and optically active substance persisted in the control solutions of glucose as well as in the glucose-muscle preparations.

The main points of interest which seem to have been emphasized by the two series of experiments include the following facts. If 20 to 60 units of insulin are added to 100 cc. of a solution of glucose in concentration of 1.0 to 0.3 per cent no change in rotatory or reducing power is detected on incubation at 37° over periods of from 1 to 5 hours. If muscle tissue is added to a solution of glucose in concentrations between 1.0 and 0.3 per cent and the solution, after incubation at 37° for a period of 2 hours, is subjected to ultrafiltration or dialysis, a slight diminution in the sugar concentration is usually demonstrable, as shown by a fall in both reducing and rotatory determinations, a fall which is presumably due to dilution by tissue fluid. Furthermore in these solutions slight irregular discrepancies seem to occur between the rotatory and reducing determinations, and these discrepancies

seem to be more prominent in the solutions which have been subjected to agitation in the water bath. The addition of insulin to these glucose-muscle preparations, in the concentration above described, has not been shown to alter materially this rotatory-reducing ratio. Finally the presence of at least one other optically active substance has been demonstrated in these solutions; namely, lactic acid.

The findings are essentially in accord with those reported by Barbour (3).

In conclusion I wish to express my thanks to Mr. J. G. Camac and Miss E. F. Herr for assistance in the analytical work and to Dr. J. H. Austin for his helpful criticism in the course of the experiments.

SUMMARY.

Ultrafiltrates and dialysates from a series of glucose solutions containing muscle tissue, both with and without insulin, have been studied over varying periods of time ranging from 1 to 48 hours.

The reducing power of these glucose-muscle tissue solutions both with and without insulin, has been found to remain constant after the effect, probably due to dilution, caused by the addition of the muscle tissue has been established.

The ratio of rotatory to reducing power in the glucose-muscle solutions both with and without insulin is influenced by slight irregular fluctuations in the rotatory values, which we suspect may be due to various optically active substances and which are noticeable in the glucose-muscle tissue solutions whether with or without insulin. In the first series of experiments, which were subjected to more prolonged contact with the muscle tissue than was the case in the second series, there is evidence of a slight rise in the rotatory-reducing value ratio up to 4 hours. This is equally evident in the glucose-muscle tissue solutions whether with or without insulin. This change is not so evident in the filtrates and dialysates of the second series of experiments when studied over a period of from 3 to 48 hours.

Marked production of acid occurs in the glucose-muscle solutions both with and without insulin and the presence of lactic acid has been found in the ultrafiltrates.

There is no evidence from these experiments to suggest that the specific rotation of glucose is altered by the addition of insulin to the glucose-muscle solution.

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INVESTIGATIONS INTO THE STANDARDIZATION AND CALIBRATION OF COLLODION MEMBRANES. I.

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INTRODUCTION.

When, in our investigations into the action of insulin *in vitro*,[1] we had occasion to determine the specific rotation angle of the glucose in a mixture containing blood, we tried to prepare the glucose in a form suitable for polarization estimations by dialysis through collodion membranes.

The collodion membranes used had to possess two qualities; namely, they must keep back the proteins and pigments of the blood with certainty, and the glucose must pass through them rapidly. Our preliminary experiments on the preparation of the membrane which was best suited to our purpose led to accurate determinations of the rate of diffusion of glucose through collodion membranes and so to the problem of standardizing them and the possibility of making uniform membranes. An account will now be given of these investigations.

HISTORICAL.

Fick (1855) was the first to use collodion membranes for studying diffusion phenomena. 5 years later closed collodion sacs were used by Schumacher. Graham (1862) introduced the term dialysis for the process by which, with the aid of membranes, he separated the diffusible crystalloids from the non-diffusible colloids.

Subsequent investigations have taught us that the sharp distinction which Graham drew between crystalloids and colloids cannot be maintained, because gradual transitions occur, as everywhere in biology. Like parchment and gelatin membranes, collodion membranes possess a porous structure, and the size of the pores determines the yield of the membrane.

[1] Lundsgaard and Holbøll (1924–25).

The smaller the diameter of the pores, the smaller are the molecules which are able to pass through the membrane.

Collodion membranes can be prepared with pores of very different sizes. They may be made so tight that crystalloids with large molecules will not go through them, and they can be made with such large pores that even the large protein molecules can slip through. Thus Gorsline in 1903 showed that crystalloids were not the only substances which could diffuse through a collodion membrane, for in 24 hours at 35°C. peptones, albumoses, albumin, starch, dextrin, and certain enzymes all dialyzed through it in a determinable amount.

In 1896 Metchnikoff and Roux introduced collodion membranes into bacteriological science in their investigations on the cholera bacillus. Since then the membranes have been used for a number of different purposes. Here may particularly be mentioned their employment in the study of the osmosis of the protein bodies (Starling, Moore and Parker, Moore and Roaf, Lillie, Loeb, Sørensen, Krogh, etc.), and their application to colorimetric determinations of the pH value of the blood (Dale and Evans, Lindhard).

As regards the shape of the membranes two types have been evolved: the sac-shaped and the flat ones. Without further entering into the advantages and disadvantages of the two forms here, it may be remarked that the flat membranes can only be used for dialysis experiments when they are mounted on a special apparatus designed for the purpose.

Earlier Attempts to Prepare Collodion Membranes of Variable Permeability.

The first successful attempt to construct a series of collodion membranes of variable permeability was made by Bechhold (1907, 1908). By impregnating filter paper with solutions containing collodion in different concentrations, he prepared membranes whose permeability varied inversely with the strength of the solution employed.

Of other methods used for preparing membranes of different permeability the one described by Schoep(1911)may be mentioned. His collodion membranes, moreover, possessed a far greater permeability than did Bechhold's. The increased permeability was obtained by adding glycerol to the collodion solution. By varying the amount of glycerol added between 2 and 10 per cent a series of membranes was obtained with a wide range of permeability.

Later, Brown (1915) developed another method of varying the permeability. The collodion membranes were first completely dried by air and then put in a mixture of alcohol and water for 24 hours at 20°C., and finally thoroughly washed in water. The higher the alcohol concentration, the more permeable were the membranes. Walpole (1915) has worked specially with flat membranes. He poured a measured quantity of the collodion

solution on a definite area of a glass plate.[2] After drying in air for some time the membranes were plunged in water. The permeability of the finished membrane decreased with the length of drying. Walpole has since recommended another procedure. According to this the collodion membrane is put in a solution of alcohol and water as soon as the ether of the solution has evaporated. The greater the concentration of alcohol, the finer are the membrane pores.

As early as 1910 Malfitano had shown that the properties of collodion membranes depended on the percentage of alcohol in the collodion solution used, the permeability rising with the alcohol concentration. Eggerth later availed himself of this observation and used the method to prepare a series of membranes with widely different permeabilities.

Lastly Krogh (1922) prepared collodion membranes of different permeability for his experiments on the osmotic pressure of the protein bodies by varying both the alcohol percentage and the length of drying.

Previous Attempts to Calibrate the Permeability of Collodion Membranes.

Having described the various methods employed for preparing collodion membranes of different permeability, if we now turn to the question of what methods have been used to demonstrate the different permeability of different membranes and particularly to measure quantitatively the yield of any given membrane, we find that this aspect of the problem has not been thoroughly investigated.

Most workers have demonstrated the different permeability of different membranes by investigating which of a series of different substances, having molecules of widely varying size, pass through, and which do not, by examining the dialysate from the substances in question after dialysis had gone on for a suitable time. The substance with the largest molecules which had passed through the membrane then gave a certain qualitative expression of the membrane's yielding capacity.

In 1908 Bechhold introduced a quantitative method for determining the permeability of membranes, one which cannot only be used to demonstrate differences in permeability, but also to find out whether a number of membranes prepared in the same manner are similar. Bechhold measured the rate of water filtration through the membrane by estimating the quantity of water which passes through 1 sq. cm. of the membrane in 1 minute at a pressure of 1 atmosphere. Krogh has since employed the same procedure and he has found uniform values for membranes prepared in the same way, but large variations for membranes made differently with respect

[2] Flat collodion membranes of exactly the same thickness throughout are best made by pouring the collodion solution on the surface of mercury, *e.g.* in a Petri plate.

to the length of drying and the percentage of alcohol in the collodion solution.

Eggerth has compared the permeability of membranes by making quantitative determinations of various substances in the dialysate after a definite interval. When all the conditions except the membranes were kept constant he found the same amount of the substances investigated for membranes prepared in the same way, but otherwise variable amounts were obtained.

Author's Investigations.

We chose Walpole's method of varying the strength of the collodion membranes by placing them in solutions of alcohol of

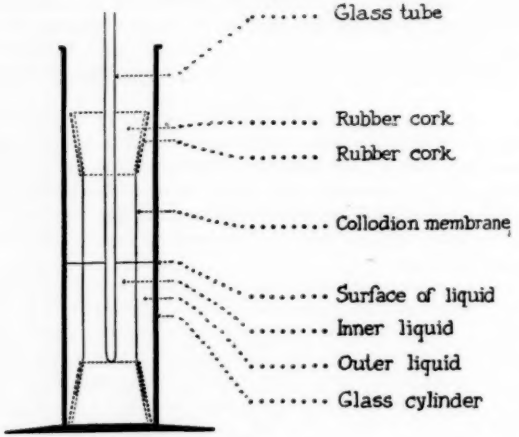

FIG. 1. Dialyzing apparatus.

different concentrations. The membranes were made in the form of a cylindrical tube, the collodion solution being poured onto a glass tube which was slowly rotating on its own axis. The glass tube was carefully freed from grease with soap solution and toluene. The solution employed was always Kahlbaum's collodion for preparing membranes. As soon as the ether in the solution had evaporated, the glass tube was put in a vessel containing alcohol of a definite concentration. After standing in the alcohol solution for 10 minutes the glass tube with the membrane was put in running water, and in a short time the membrane could be easily

detached. It was then mounted as shown in Fig. 1 from which the arrangement of the dialysis experiments will be seen.

The collodion membranes used in the following experiments had a diameter of 24 mm. The lower end of the cylindrical membrane tube was closed with a rubber cork, to which the membrane was tightly fastened with a piece of rubber tubing. After the inner liquid was introduced the upper end of the membrane tube was closed (not air-tight) in the same way with a rubber cork, through which a glass tube, serving as a support for the membrane, passed down to the bottom. For the experiments the membrane is put in a glass cylinder which is chosen of such a diameter that the volume of outer liquid to be used reaches to the same height outside the collodion membrane as the inner liquid does inside.

Experiments on the Determination of the Diffusion Coefficient of Glucose through a Given Membrane.

In the first instance the law governing the passage of glucose from a solution through a given membrane was investigated, using collodion membranes dried in 70 per cent alcohol. The arrangement will be seen from Fig. 1. As inner liquid a glucose solution of a definite strength was used; the outer liquid was distilled water. The volumes of the inner and outer liquids were the same in all the experiments, namely 40 cc. The surfaces of the liquids therefore stood at the same height. As the inner tube had a diameter of 6 mm. the surface of the membrane through which dialysis took place can be estimated. This was 71 sq. cm.

The experiments were performed in the following manner. Samples of the inner and outer liquids were pipetted off for glucose determinations at suitable intervals of time. At first samples were taken every 15 minutes, later not so frequently. Before removing the sample the liquids were thoroughly stirred. The glucose content was determined by Hagedorn and Norman Jensen's method. The volumes of the inner and outer liquids were only slightly diminished during the experiment as a result of the trifling amount of liquid withdrawn for analysis (0.2 cc.), so that the surface through which diffusion took place was only slightly less at the end of the experiment. All the experiments were done at 20°C., as the diffusion of a given substance through a given membrane is dependent on the temperature and increases with it.

For the first experiments a collodion membrane was employed, as stated, which was dried in 70 per cent alcohol. Three experiments were made with the same membrane in which the glucose concentrations of the inner liquid at the beginning of the experiment were 0.3, 0.2, and 0.1 per cent respectively. The results are given in Table I, and the curves representing them, in Fig. 2. They show that the concentration of the outer liquid steadily increases in the same proportion as that of the inner liquid decreases, until, after 3 hours have elapsed, the same concentration

TABLE I.

Results of Diffusion of Glucose through a Collodion Membrane Dried in 70 Per Cent Alcohol.

Time after dialysis was started.	Experiment I.		Experiment II.		Experiment III.	
	Glucose in outer liquid.	Glucose in inner liquid.	Glucose in outer liquid.	Glucose in inner liquid.	Glucose in outer liquid.	Glucose in inner liquid.
min.	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
15	0.053	0.245	0.035	0.164	0.017	0.083
30	0.086	0.213	0.057	0.144	0.028	0.073
45	0.107	0.194	0.072	0.127	0.036	0.063
60	0.122	0.175	0.082	0.118	0.041	0.060
90	0.137	0.162	0.092	0.109	0.046	0.055
120	0.145	0.155	0.097	0.104	0.048	0.052
150	0.148	0.153	0.099	0.102	0.049	0.052
180	0.150	0.150	0.100	0.100	0.050	0.050
Glucose in inner liquid when experiment was started, *per cent*.....	0.300		0.200		0.100	

is found on both sides of the membrane. On comparing the concentrations of the inner and outer liquids it will be seen that no appreciable shifting of the volumes of the inner and outer liquids has occurred in spite of the difference in osmotic pressure present when the experiment started.

Since the same volume of liquid was used in all the experiments, namely 40 cc., the liquid height was identical in all of them, so that no difference in the rate of diffusion can have occurred from this cause.

We have made a mathematical calculation from these results. The diffusion of the glucose through the collodion membrane under the given conditions, where the inner and outer liquid volumes are the same, proceeds in accordance with the following equation:

$$\frac{dx}{dt} = c\left(\frac{K}{2} - x\right)$$

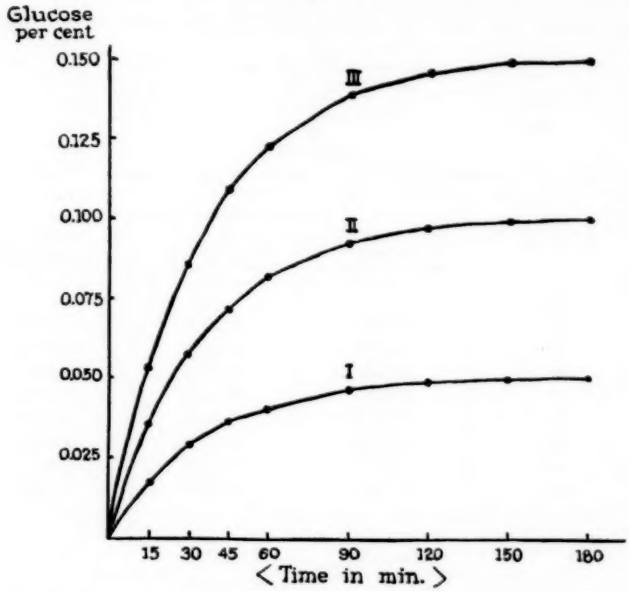

FIG. 2. Glucose concentrations in the outer liquid from the experiments in Table I. Curve I, 0.1 per cent; Curve II, 0.2 per cent; Curve III, 0.3 per cent glucose in the inner liquid when the experiment was started.

where K is the primary concentration in the inner liquid, x is the increase in concentration in the outer liquid in the time t, and c is the diffusion coefficient of the glucose for the given membrane. On integrating this equation and solving with respect to the diffusion coefficient c we obtain:

$$c = \frac{1}{t} \log_e \frac{K}{K - 2x}$$

In Table II the values of c calculated from the experimental results in Table I are given, the time being measured in minutes. As the results show, very constant values are obtained by this method of calculation. The values are naturally most uniform at the beginning of the experiment. Later on, when the diffusion is slower and the increment x therefore slight, the coefficient, of course, deviates more. If we take the mean value of the determinations of the diffusion coefficient during the 1st hour we obtain:

For concentration 0.1 per cent: $c = 0.0121$.
" " 0.2 " " " $= 0.0123$.
" " 0.3 " " " $= 0.0123$.

TABLE II.

Diffusion Coefficient of Glucose Calculated from Results in Table I.

Time after dialysis was started.	Diffusion coefficient of glucose.		
	Experiment I. 0.3 per cent glucose.	Experiment II. 0.2 per cent glucose.	Experiment III. 0.1 per cent glucose.
min.			
15	0.0126	0.0125	0.0120
30	0.0123	0.0122	0.0119
45	0.0121	0.0122	0.0122
60	0.0121	0.0124	0.0124
90	0.0118	0.0122	0.0122
120	0.0123	0.0127	0.0116
150	0.0126	0.0133	0.0113
180	0.0121	0.0122	0.0122
Mean of first four values.....	0.0123	0.0123	0.0121

By this mode of procedure and calculation, therefore, a numerical expression for the diffusion of glucose through a given collodion membrane has been obtained. *The yielding capacity of a membrane is thus expressed by a number.* The method employed is fairly simple: When the volumes of the inner and outer liquids are kept equal, it is only necessary to determine the concentration of the inner liquid at the beginning of the experiment and make three or four determinations of the concentration of the outer liquid during the 1st hour. As is evident from the values in Table II, the diffusion coefficient is independent of the initial concentration of the glucose in the inner liquid within the limits 0.1 to 0.3

TABLE III.

Dialysis Experiment. Initial Concentration of Inner Liquid 1.6 Per Cent Glucose.

Time after dialysis was started.	Glucose in outer liquid.	Diffusion coefficient c.
min.	*per cent*	
15	0.275	0.0122
30	0.451	0.0120
45	0.566	0.0119
60	0.660	0.0126
90	0.734	0.0120
120	0.768	0.0116
150	0.787	0.0119
180	0.798	

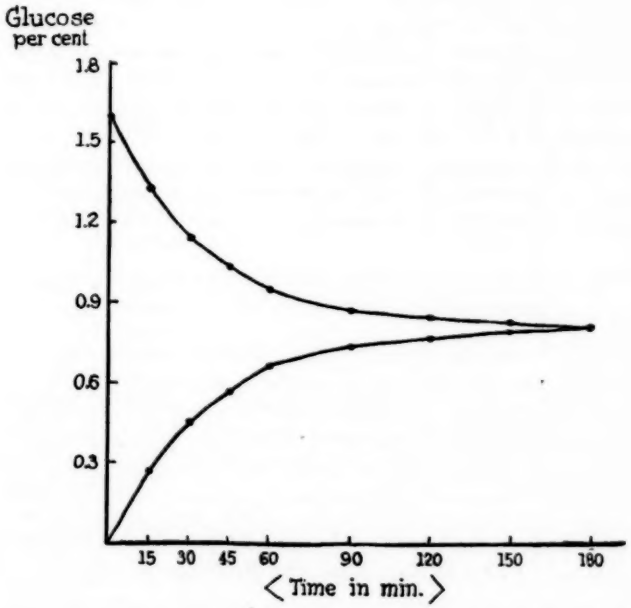

FIG. 3. Dialysis experiment. Concentration of glucose in the outer and inner liquids.

per cent, which were investigated. Finally an experiment was made with the same membrane in which the initial concentration was much higher, namely 1.6 per cent. The results are recorded in Table III and Fig. 3. They show that the diffusion coefficient has the same value at this concentration.

TABLE IV.

Diffusion Coefficient of Glucose for Ten Collodion Membranes Immersed in 70 Per Cent Alcohol.

Membrane No.	Diffusion coefficient c for four first periods.	Diffusion coefficient c. Mean value.	Membrane No.	Diffusion coefficient c for four first periods.	Diffusion coefficient c. Mean value.
1	0.0125	0.0124	6	0.0115	0.0118
	0.0120			0.0119	
	0.0124			0.0120	
	0.0126			0.0119	
2	0.0129	0.0130	7	0.0125	0.0127
	0.0133			0.0125	
	0.0131			0.0129	
	0.0128			0.0128	
3	0.0125	0.0126	8	0.0127	0.0127
	0.0125			0.0126	
	0.0126			0.0129	
	0.0127			0.0125	
4	0.0122	0.0123	9	0.0127	0.0128
	0.0124			0.0128	
	0.0120			0.0128	
	0.0125			0.0128	
5	0.0139	0.0134	10	0.0115	0.0115
	0.0135			0.0118	
	0.0132			0.0114	
	0.0131			0.0114	

Experiments on the Degree of Uniformity with Which Membranes of a Definite Type Can Be Prepared.

As we were now able to express the yield of a membrane by a numerical value by investigating the diffusion of a glucose solution through it, the way was opened up for the task of estimating the

diffusion coefficient of a series of membranes made by the same method in order to discover how uniform such membranes were.

Ten collodion membranes were prepared for the purpose, and all of them were immersed in 70 per cent alcohol. They were then mounted as previously described. The glucose solutions employed had concentrations varying between 0.2 and 0.4 per cent. In Table IV the calculated values of the diffusion coefficient c for the first four periods and the mean values obtained from them, are recorded.

As appears from the table the mean values of the diffusion coefficients for the membranes examined varied between 0.0115 and 0.0134. The mean value for the ten membranes investigated was 0.0125. The greatest deviation from the mean value was therefore 8 per cent. The values entered in the second column of the table, calculated from the results in the first four experimental periods, deviate still less from one another in the case of the individual membranes, the greatest variation from the mean value being 3.8 per cent. The small deviations of the value c for the ten different membranes can therefore hardly be explained as experimental errors, but must be due to real differences between the membranes which, moreover, one would expect to find. The variations detected are however so slight that it can be stated *that uniform membranes can be prepared by the method adopted.*

Investigations into the Significance of the Concentration of Alcohol Solution Used in Preparation of Membranes.

All the membranes used in the experiments described so far were, as mentioned, immersed in 70 per cent alcohol. We next investigated in what way a change in the concentration of the alcohol would alter the yielding capacity of the membrane. In these experiments the membrane was put in 80 or 90 per cent alcohol.

The actual experiments were done in exactly the same manner as those previously described, so that the membrane surface through which dialysis took place was the same. The results are recorded in Table V and graphically represented by Fig. 4. The experiments were made with a 0.2 per cent glucose solution, and for the sake of comparison the results of the experiment with the

TABLE V.

Dialysis Experiments with Three Membranes Immersed in 70, 80, and 90 Per Cent Alcohol Respectively. Initial Glucose Concentration in the Inner Liquid in All Experiments 0.2 Per Cent.

Time after dialysis was started.	70 per cent alcohol.		80 per cent alcohol.		90 per cent alcohol.	
	Glucose in outer liquid.	Diffusion coefficient.	Glucose in outer liquid.	Diffusion coefficient.	Glucose in outer liquid.	Diffusion coefficient.
min.	*per cent*		*per cent*		*per cent*	
15	0.035	0.0125	0.028	0.0095	0.018	0.0057
30	0.057	0.0122	0.048	0.0097	0.033	0.0058
45	0.072	0.0122	0.063	0.0096	0.045	0.0058
60	0.082	0.0124	0.073	0.0094	0.055	0.0058
90	0.092	0.0122	0.086	0.0095	0.070	0.0058
120	0.097	0.0127	0.093	0.0096	0.080	0.0058
150	0.099	0.0133	0.096	0.0093	0.086	0.0057
180	0.100	0.0123	0.098	0.0094	0.091	0.0057

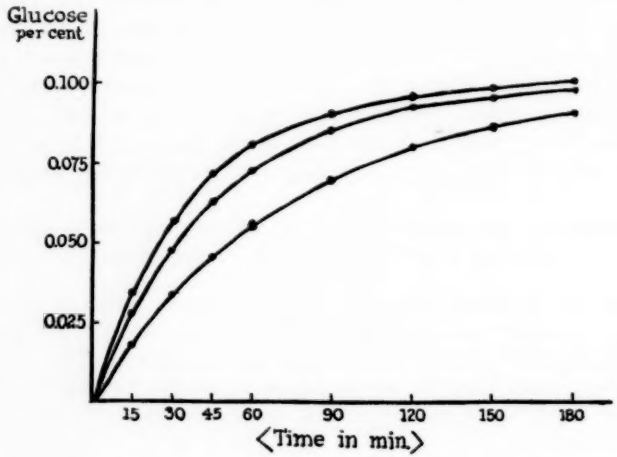

FIG. 4. Concentration of glucose in the outer liquid in the experiments recorded in Table V.

same glucose concentration and a membrane which had been immersed in 70 per cent alcohol are given. It will be seen that the dialysis proceeded more slowly the higher the concentration of the

alcohol solution used. This can be expressed numerically if the diffusion coefficient is calculated. This value was determined with the help of the equation given previously and is recorded with the results of the experiments in Table V.

If the mean value of the diffusion coefficient c for the first four periods is taken the following values are obtained:

Alcohol concentration.	Mean value of c for first four periods.
per cent	
70	0.0123
80	0.0096
90	0.0058

In order to find out whether membranes of these types could be made with just as great exactitude as those previously examined which were prepared with 70 per cent alcohol, the diffusion coefficient of glucose for three membranes immersed in 80 per cent alcohol and three membranes immersed in 90 per cent alcohol was determined. From the results of the dialysis experiments, which were carried out with precisely the same technique as before, the diffusion coefficient was calculated, and the mean values for the first four periods are recorded in Table VI. As the results show, it was also possible in these cases to prepare uniform membranes of the given standard type.

Investigations into the Influence of Time on the Constancy of the Membrane.

All the previously described experiments were done with collodion membranes which had either been made on the same day that the experiment took place or the preceding day. After being made, and until it was used, the membrane had been kept in distilled water with a trace of toluene added. At no time were the membranes allowed to dry, as they then quickly lose their properties.

It is of practical importance to know how long the membranes keep their characters unchanged. To settle this point the diffusion coefficient of glucose for five collodion membranes immersed in 70 per cent alcohol was determined immediately after they were

made and after keeping for a week. The results are given in Table VII.

It will be seen that only very small changes in diffusion can be detected, and as these variations do not all go in the same direction the investigation proves that the membranes can quite well be kept in the way described for 1 week and yet retain their properties intact. How long the membranes keep constant as regards their yielding capacity beyond this period was not investigated, be-

TABLE VI.

Diffusion Coefficient of Membranes Prepared with 80 and 90 Per Cent Alcohol.

Membrane No.	Alcohol concentration.	Diffusion coefficient.
	per cent	
1	80	0.0095
2	80	0.0093
3	80	0.0092
4	90	0.0057
5	90	0.0057
6	90	0.0059

TABLE VII.

Diffusion Coefficient of Glucose for Five Collodion Membranes Immediately and 1 Week after Making.

Membrane No.	Diffusion coefficient.	
	Immediately.	After 1 week.
1	0.0133	0.0132
2	0.0123	0.0117
3	0.0125	0.0125
4	0.0119	0.0115
5	0.0127	0.0130

cause, on practical grounds, it would not be necessary to keep them for longer than a week.

Investigations into the Relation between Rate of Diffusion and Membrane Surface and Volume of the Liquids.

All the experiments described above were, as stated, carried out with collodion membranes of the same width, and with the same volume of liquid inside and outside, so that the surface through

which diffusion took place was the same in every experiment. It is known that the rate of diffusion is dependent on the relation of the size of the surface to the volumes of the liquids. In order to investigate to what extent the diffusion could be accelerated by increasing the surface in relation to the volume, some experiments were made with membranes of a different width. In setting up the apparatus the diameter of the internal tube (see Fig. 1) was made considerably larger.

The diameter of the membrane was 30 mm. and that of the internal tube 24 mm., so that the distance between the membrane and the inner aspect of the glass tube was only 3 mm. The volume of the inner liquid was 40 cc., so that the membrane surface through which diffusion took place is found on calculation to be 148 sq. cm., thus a little over twice as large as in the case of the membrane previously used. The outer glass cylinder was chosen with such an internal diameter that the outer liquid, whose volume was also 40 cc., rose to the same height on the collodion membrane outside as did the liquid inside. The experiments themselves were otherwise performed in the same manner as the previous ones. The results of the dialysis of a 0.2 per cent glucose solution through a membrane treated with 70 per cent alcohol are recorded in Table VIII and in Fig. 5, where the results of an identical experiment with the previously employed arrangement of the membrane are also given for comparison.

The rate of diffusion is, therefore, considerably increased by this arrangement. Equilibrium between the glucose contents of the inner and outer liquids has thus taken place in 1½ hours, while it takes 3 hours with the previous arrangement.

The reason that the values of the diffusion coefficient deviate from one another, particularly in the later periods, rather more than they did in the previous experiments, is that only small changes in the concentration occur in the later periods, so that an insignificant experimental error can more easily be detected.

Besides the membrane referred to, three others prepared in exactly the same way were examined. As the mean value for the diffusion coefficient of the single membrane the values obtained in the two first periods were used. The mean value for the diffusion coefficient of all four membranes was 0.0300. The increase in the rate of diffusion was due naturally first of all to the enlarge-

TABLE VIII.

Diffusion of 0.2 Per Cent Glucose Solution through Collodion Tubes of Different Widths. Volume of Liquids in Each Experiment Was 40 Cc. Internally and Externally.

Time after dialysis was started.	Width of membrane 30 mm. Inner tube 24 mm.		Width of membrane 24 mm. Inner tube 6 mm.	
	Glucose in outer liquid.	Diffusion coefficient.	Glucose in outer liquid.	Diffusion coefficient.
min.	*per cent*		*per cent*	
15	0.065	0.0304	0.035	0.0125
30	0.087	0.0295	0.057	0.0122
45	0.096	0.0310	0.072	0.0122
60	0.098	0.0283	0.082	0.0124
90	0.100		0.092	0.0122
120	0.100		0.097	0.0127
150			0.099	0.0133
180			0.100	

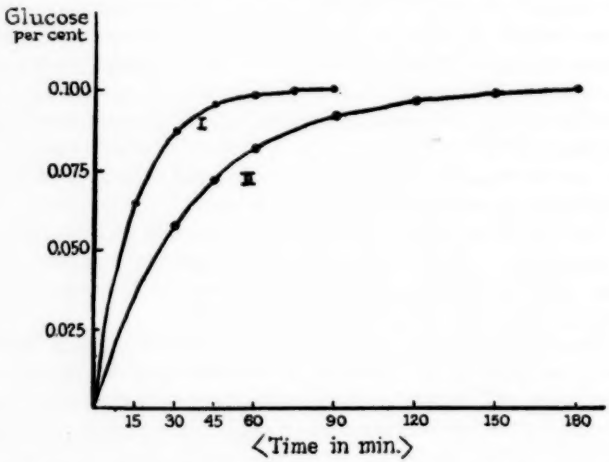

FIG. 5. Glucose concentration of the outer liquid of the experiments recorded in Table VIII.

ment of the surface of the membrane, but probably at the same time to the fact that the horizontal depth of the liquid layer on each side of the membrane was less. Both the inner and outer

liquids were carefully stirred before each sample was taken, but it can hardly be denied on this account that diffusion in the inner and outer liquids themselves can influence the rate of diffusion through the membrane.

With the idea of possibly casting some light on this question an experiment was made with a membrane like that described in Fig. 1, where a stream of air was pumped through the outer and inner liquids during the entire experiment. The results are given in Table IX. Details of two experiments with the same glucose concentration, 0.2 per cent, are given. One experiment was conducted as usual, while in the other air was blown through the liquids. As the results show there was a slight increase in the rate of diffusion. This increase, however, was so small that the

TABLE IX.

Rate of Diffusion of Glucose through the Same Membrane with and without Blowing Air through the Liquids.

Time after dialysis was started.	Without blowing air through.		With blowing air through.	
	Glucose in outer liquid.	Diffusion coefficient.	Glucose in outer liquid.	Diffusion coefficient.
min.	*per cent*		*per cent*	
15	0.035	0.0125	0.038	0.0138
30	0.057	0.0122	0.062	0.0140
45	0.072	0.0122	0.077	0.0142
60	0.082	0.0124	0.086	0.0142

method can scarcely be of practical importance when it is desired to increase the rate of diffusion. For this purpose it will be much more advantageous, as shown by Table VIII, to use an arrangement where the membrane surface is large in relation to the volumes of the liquids.

SUMMARY.

1. The laws governing the passage of glucose through a given collodion membrane were investigated. It appears that when the volumes of the liquids on both sides are the same the diffusion follows the equation:

$$\frac{dx}{dt} = c\left(\frac{K}{2} - x\right)$$

where K is the initial concentration of the inner liquid, x is the increase in concentration in the outer liquid in the time t, and c is the diffusion coefficient of glucose for the given membrane.

2. The diffusion coefficients of a series of collodion membranes were determined, which in accordance with Walpole's instructions, had been immersed in solutions of alcohol. The solutions employed contained 70, 80, and 90 per cent alcohol. The diffusion coefficient was found to be the same for membranes prepared in the same manner but varied according to the concentration of alcohol used, the diffusion coefficient decreasing with rising alcohol concentration.

3. A useful method has thus been devised for determining the yield of a given membrane and proof has been furnished that it is possible to standardize collodion membranes by varying the strength of the solution of alcohol in which they are immersed.

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STUDIES IN CARBOHYDRATE METABOLISM.

VI. TECHNIQUE APPLIED IN DETERMINING THE PRESENCE OF NEW-GLUCOSE IN VARIOUS BIOLOGICAL FLUIDS.

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INTRODUCTION.

In a series of publications we have demonstrated the presence of a special form of glucose in various biological fluids. This sugar form is not identical with the usual α,β-glucose, because it has another specific rotatory power. As a *preliminary* name for this sugar form we have proposed the term new-glucose.

The presence of new-glucose has been demonstrated in: (*a*)

dialysate from blood of normal individuals (1); (*b*) dialysate from blood of diabetics drawn 2 hours after injection of insulin (whereas it was not found in the majority of severe diabetics) (2); (*c*) in spinal fluid, pleural exudate, and edema fluid from patients with normal carbohydrate metabolism (3). Furthermore it has been shown that new-glucose can be produced *in vitro* from α,β-glucose by addition of muscle tissue and insulin to a glucose solution (4). Hitherto new-glucose has been found only in fluids which also contain α,β-glucose.

The demonstration of new-glucose in a solution is based on two facts: (1) that it has a lower specific rotatory power than has ordinary α,β-glucose, and (2) that its curve of mutarotation runs a characteristic course different from the curve of mutarotation of either α-glucose or β-glucose. For details of mutarotation of sugars see Paper II of this series (5).

The procedure applied by us in demonstrating the presence of new-glucose in fluids is therefore built up on the methods for determining the reducing power and the rotatory power of glucose solutions. The main features of our technique are briefly described in our previous publications. In this article the details will be given.

A. Polarimetric Procedure.

All the determinations of the concentration of glucose in a solution by means of polarimetry are in principle identical: The concentration of glucose is calculated from the size of the angle which expresses how much the plane of the polarized light is changed, when the light passes through a glucose solution in a tube which is placed in a polarimetric apparatus between two Nicol's prisms. The accuracy of polarimetric determinations is dependent on various factors: (1) the kind of polarimetric apparatus used; (2) the length and the width of the polarimetric tube; (3) the source of light; (4) the transparency of the fluid to be analyzed; (5) the concentration of the glucose in the solution; and (6) to what degree the observer's eye can make two light-fields match each other.

In all our determinations we have used a Schmidt-Haensch polarimeter, the scale of which is divided into Ventzke degrees. On the scale 0.1° Ventzke can be read directly. 1° Ventzke on

the scale equals 0.03448 ordinary circle degrees. As source of light a 100 watt electric lamp has been used. In order to obtain monochromatic light it is filtered through a layer of a 3 per cent solution of potassium dichromate 3 cm. deep. We prefer this source of light to sodium light, which ordinarily is used in polarimetric determinations, because it is possible in this way to obtain a much stronger light, which gives increased exactness of the readings. In order to make sure that the light which we use has a correct wavelength the specific rotatory power was determined for a series of solutions of α,β-glucose in equilibrium. As to the transparency of the solution, we want to emphasize that even the faintest turbidity of the fluid decreases the exactness of the reading to a very great extent. For that reason we have always used solutions absolutely clear. Our procedure in producing such solutions is described below.

Length and Diameter of the Polarimetric Tube.

The degree to which the plane of the light is turned by a given solution is proportional to the length of the polarimetric tube. In all our determinations we have used tubes 20 cm. long. The diameter of the polarimetric tube has, on the other hand, no influence on the degree of rotation of the plane for a given solution of glucose. If, however, the diameter of the tube is diminished below a certain value the reading becomes less accurate. The polarimetric tubes generally used have an internal diameter of 8 to 9 mm. However, in most instances it was of importance to us to use the smallest possible amount of fluid. For that reason we have determined how narrow the tube can be before the accuracy of the reading is lessened. In absolutely clear solutions and by using glass tubes, the smallest diameter, which can be used without diminishing the clearness of the field, is 6 mm. Consequently the smallest amount of fluid used for each of our determinations is about 6 cc.

Adaptation of the Eye of the Observer.

The main error in the source of polarimetric reading lies in the eye of the observer. It is particularly important that the eye is adapted for the clearness of the field which is to be read. If one

starts reading immediately after entering the dark-room in which the polarimetric apparatus is placed, it is almost inevitable that the first readings are incorrect and cannot be used. We have, therefore, before our determinations, waited 2 minutes in the dark-room in order to adapt the eye. It is possible that the adaptation time is not the same for all observers. Ordinarily the eye becomes increasingly capable of distinguishing differences in light the more determinations one makes at the same seance. However, if one makes too many readings the eye gets tired, and the determinations become inexact.

Number of Readings in Each Determination.

The exactness of a polarimetric determination increases with the number of readings. *Therefore, one or a few readings do not give, in determinations like ours, sufficient exactness.* In every determination the rotation value for the solution must be obtained as the average of a certain number of readings, and these readings must agree well. The number of readings used by us in each determination has varied from 10 to 40 according to the amount of glucose in the solution. If the concentration is 0.5 per cent or more, 10 readings are sufficient. For solutions between 0.2 and 0.5 per cent glucose, 20 readings are necessary in each determination, and if the concentration of glucose in the solutions is below 0.2 per cent, 40 readings are necessary. This point is very important.

Examples of Readings.

In Table I we have given all the directly read values in Ventzke degrees from three determinations of the rotatory power of a 0.100 per cent solution of α,β-glucose. This concentration of glucose corresponds fairly well to the normal concentration of sugar in the blood of a normal fasting individual. Each determination is, as stated, the result of 40 readings. All the readings are made on the same day and under constant temperature. 40 readings require about 15 minutes. Between the three sets of readings the observer has paused for 10 minutes, and the eye is adapted each time.

The average values for each of the 40 readings in Table I are for the three determinations:

I. 0.3000° Ventzke.
II. 0.3050° "
III. 0.3075° "

If the concentration of α,β-glucose, which has a specific rotatory angle of + 52.5 per cent, is calculated from the above values, the following glucose percentages are obtained:

	Glucose. *per cent*
I.	0.0985
II.	0.1002
III.	0.1010

TABLE I.

Three polarimetric determinations each consisting of 40 readings expressed in Ventzke degrees. Polarimetric tube 20 cm. long. 0.1 per cent solution of α,β-glucose.

1st determination.				2nd determination.				3rd determination.			
0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3
0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.4	0.3	0.4	0.3	0.3
0.3	0.3	0.3	0.2	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3
0.4	0.3	0.3	0.3	0.3	0.4	0.3	0 3	0.3	0.3	0.3	0.4
0.3	0.3	0.2	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3
0.3	0.4	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3
0.2	0.3	0.3	0.4	0.3	0.3	0.3	0.3	0 4	0.3	0.3	0.3
0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.2	0.3	0.3	0.3	0.3
0.3	0.3	0.3	0.3	0.4	0.3	0.3	0.3	0.3	0.3	0.3	0.3
0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3
Average..0.3000°				0.3050°				0.3075°			

The deviations from the correct value (0.1 per cent glucose solution) are in these three determinations 0.0015, 0.0002, and 0.0010 per cent glucose. The maximum deviation is 1.5 per cent of the correct value.

The error in polarimetric reading is, as previously mentioned, constant. In determinations performed with an accuracy equal to the readings in Table I the absolute deviation from the correct value is consequently not more than 2 in the third place, if the concentration of glucose in the solution is given in per cent. If

the error is expressed in percentage of the correct value, however, it is naturally dependent on the glucose solution, the error being greater the smaller the concentration of the glucose.

In Table II is given the relative error corresponding to different concentrations of glucose. For a 0.1 per cent glucose solution the maximum error is 1.5 per cent. For a 0.05 per cent solution it is 3 per cent, and for a 0.03 per cent solution 5 per cent. We have previously mentioned that a glucose concentration of 0.1 per cent corresponds fairly well with the normal fasting value of the blood sugar, and a 0.03 per cent glucose solution corresponds fairly well with the smallest glucose solution which we have determined.

TABLE II.

Maximum error by the polarimetric determination of various glucose solutions if readings are made as stated in Table I.

Concentration of glucose solution used.	Maximum error expressed in per cent of glucose concentration.
per cent	
0.1	1.5
0.05	3
0.03	5

B. Determination of the Mutarotation.

The mutarotation curve for new-glucose runs, as previously stated, a characteristic course (see Fig. 1 (3)). The mutarotation is as a matter of fact the best proof for the presence of new-glucose in a solution. In our previously published investigations we have therefore *not been satisfied by comparing the rotation and reduction values in the solution immediately after its preparation, but we have in all our determinations followed the increasing rotation value until it became constant. Then the reduction value was again determined in order to make sure that no change in the glucose concentration had taken place.* The course of the mutarotation curve can be determined only if the solution remains absolutely clear. All the different determinations must necessarily be performed at the same temperature. In Tables III, IV, and V we shall give three examples of polarimetric readings *taken from previous publications.* The readings may serve not only as examples of readings, like the figures in Table I, but they also give the first and the last deter-

mination of the rotation, thus giving the two end-points of the curve for the mutarotation. The first series of readings is made immediately after the preparation of the fluid, and the second series of readings is the last reading during the period of mutarotation. They are in all the examples made 48 hours after the first readings. After that time the rotation power remained constant and was equal to the reduction value which was unchanged from the first determination. These three examples therefore show how great differences one may have between the first and the last

TABLE III.

Determination of the rotation power of the dialysate from blood. Reduction value 0.084 per cent glucose. Each determination consists of 40 readings expressed in Ventzke degrees. First set of determinations made immediately after the dialysis, second set of determinations 48 hours later, when the mutarotation has ceased.

1st determination.				2nd determination.			
0.2	0.1	0.2	0.1	0.3	0.3	0.3	0.2
0.2	0.1	0.2	0.2	0.2	0.3	0.2	0.3
0.1	0.1	0.1	0.1	0.3	0.2	0.2	0.3
0.2	0.2	0.1	0.1	0.3	0.3	0.2	0.2
0.1	0.2	0.2	0.2	0.2	0.2	0.3	0.3
0.1	0.1	0.1	0.2	0.2	0.2	0.2	0.3
0.1	0.2	0.2	0.1	0.2	0.2	0.3	0.2
0.2	0.2	0.1	0.2	0.3	0.3	0.2	0.3
0.1	0.1	0.2	0.1	0.2	0.3	0.3	0.2
0.2	0.1	0.1	0.1	0.3	0.2	0.2	0.3
Average.....0.145°				0.250°			

polarimetric determination. In some instances the differences have been smaller, in other instances greater. We have selected these three sets of readings as examples of our determinations in (1) dialysate from blood, (2) spinal fluid which does not need to be dialyzed, and (3) dialysate from a solution of new-glucose made by addition of insulin and muscle tissue to a solution of α,β-glucose.

Dialysate from Blood.

In Table III are given the polarimetric readings from the dialysate from normal blood. The example is taken from Case 8 A, Table II, Paper III, p. 331 (1). The reduction value equalled

0.084 per cent glucose and remained constant during the period of mutarotation.

The average value for the 40 readings in the first determination is 0.145° Ventzke, and for the last 40 determinations the average is 0.250° Ventzke. The difference between the average values of the two determinations is consequently 0.105° Ventzke. If we assume that the sugar in both instances had a specific rotation value of +52.5° the average values for the first and second determinations correspond to 0.048 per cent glucose and 0.082 per cent glucose respectively. The difference between these two values

TABLE IV.

Two determinations of the rotating power of spinal fluid, each consisting of 40 readings, expressed in Ventzke degrees. First set of readings made immediately after the spinal puncture, second set made 48 hours later, when the mutarotation has ceased. The reduction power of spinal fluid equals 0.083 per cent glucose.

1st determination.				2nd determination.			
0.1	0.1	0.1	0.1	0.2	0.3	0.2	0.3
0.1	0.1	0.2	0.1	0.3	0.2	0.3	0.2
0.1	0.1	0.1	0.1	0.2	0.3	0.2	0.3
0.2	0.1	0.1	0.1	0.2	0.2	0.3	0.2
0.1	0.1	0.2	0.1	0.2	0.3	0.2	0.3
0.1	0.2	0.1	0.2	0.3	0.3	0.3	0.2
0.1	0.1	0.1	0.1	0.3	0.2	0.2	0.2
0.1	0.1	0.1	0.1	0.3	0.2	0.3	0.3
0.1	0.1	0.1	0.1	0.2	0.3	0.2	0.2
0.1	0.1	0.1	0.1	0.2	0.2	0.2	0 2
Average.....0.1125°				0.2425°			

is 0.034 per cent. The greatest deviation in the two series of 40 readings has, however, a value corresponding to only 0.0015 per cent. *The difference between the two values is therefore more than twenty times greater than the maximum error in each of the two sets of 40 readings.*

Spinal Fluid.

In Table IV are given the polarimetric readings of spinal fluid from a patient with normal carbohydrate metabolism (Spinal Fluid III, Table I, p. 365 (3)). The rotation values recorded in the table were read immediately after the fluid was drawn from

the spinal canal and after 48 hours when the mutarotation ceased. The intervening readings are not given. The reduction value of the fluid was 0.083 per cent glucose and remained constant during the 48 hours.

The average values for the two sets of 40 readings are 0.1125° and 0.2425° Ventzke respectively. If as in the previous example, we assume a specific rotation power for the glucose in the solution

TABLE V.

Polarimetric determination of the rotating power of dialysate from an experiment *in vitro* on the influence of insulin and muscle tissue of α,β-glucose. Each determination consists of 20 readings, made by two observers. First set of readings made immediately after the dialyzing, second set 48 hours later, when the mutarotation has ceased. Reduction power of dialysate equals 1.100 per cent glucose.

1st determination.		2nd determination.	
A.G.	S.A.H.	A.G.	S.A.H.
2.6	2.6	3.3	3.3
2.7	2.7	3.3	3.4
2.7	2.6	3.4	3.3
2.7	2.7	3.3	3.4
2.7	2.7	3.4	3.4
2.7	2.7	3.3	3.3
2.7	2.7	3.4	3.3
2.8	2.7	3.3	3.4
2.7	2.7	3.4	3.4
2.7	2.7	3.4	3.3
2.70	2.68	3.35	3.35
Average.....2.69°		3.35°	

of 52.5 per cent, the glucose concentrations corresponding to the two readings are 0.037 and 0.080 per cent respectively. The difference between the two glucose concentrations calculated in such a manner is 0.043 per cent glucose, the value of the difference *is about thirty times the greatest reading error (0.0015 per cent) found in the 40 readings.*

Mutarotation of New-Glucose Produced in Vitro from α,β-Glucose.

In Table V are given the polarimetric readings in an experiment from investigations of which only a preliminary report is published

(6). The readings in Table V show the mutarotation and its determination in experiments where new-glucose is artificially produced, as was done in Paper I of this series (4). The reason why we have taken this experiment and not one from our first publication is that each half of the two sets of readings was done by two different observers independently of each other. Corresponding to what we have previously stated as to the number of readings necessary, we have made only 20 readings in each determination of these experiments. The experiment was made in the following manner:

A guinea pig was decapitated and 15 gm. of muscle tissue were immediately removed and cut in very small pieces. Sterile precautions were observed throughout. These 15 gm. of muscle tissue, 20 units of insulin (Scandinavian units, see below) were added to 200 cc. of a 2.5 per cent isotonic glucose solution, made as described below, then the solution was shaken for 2 hours at 37°C. in a shaking machine. At that time a sample was introduced into the dialyzing apparatus (see below); after 1½ hours dialysis at 20°C. the dialysate was removed. Immediately after the removal of the dialysate its reduction value was 1.100 per cent glucose, and the same reduction value was found after 48 hours. At the same time the first reduction value was determined, the dialysate was introduced into the polarimetric tube, and a series of readings was made. Then the rotation value was followed at intervals during the next 48 hours until the value became constant, when the last reduction value was determined.

In Table V are given the first and the last series of readings. The figures show that the readings of the two observers, who were both trained, agree closely. If the concentration of glucose is calculated under the assumption that the specific rotation value is +52.5°, the two determinations correspond to 0.883 per cent glucose and 1.100 per cent glucose respectively, and the latter value corresponds to the reduction value.

C. Filling the Polarimetric Tubes.

In filling the polarimetric tubes it is necessary to take great care that no air gets into the tube. We do it in the following way: The tube is held vertically, and the end-piece of one end of the tube is removed. By means of a long narrow glass pipette, which will reach the bottom of the polarimetric tube, the fluid is introduced. The tube is filled so that a convex layer of fluid comes

up over the upper part of the tube. Then the end-piece is slid on and fastened with the nut. Sterile precautions were observed throughout. In order to prevent glycolysis, 2 *pro mille* sodium fluoride is always added to the solutions. During the mutarotation period the tubes are kept at constant temperature in order to prevent air being sucked into the tube by contraction of the fluid.

D. Determination of the Reduction Value of the Glucose.

The reduction values of the glucose are determined after the method of Hagedorn and Norman Jensen. This method is based on the principle that in alkaline solutions and by boiling, glucose reduces potassium ferricyanide to potassium ferrocyanide. The non-reduced amount of potassium ferricyanide is determined by iodometric titration with N/200 thiosulfate solution. Hagedorn and Norman Jensen have devised a procedure for precipitation of the protein substances in the blood when the blood sugar is to be determined. This protein precipitation is not necessary in our determinations, because all the glucose solutions, in which we at the same time determine reduction and rotation, are protein-free. The solution of potassium ferrocyanide can therefore be added directly to the glucose solution. The volume of each sample for analysis is always 12 cc. Care is taken that each sample of glucose solution has about the same amount of glucose; namely, 0.15 mg. of glucose. If this is not the case the solution is sufficiently diluted before the 12 cc. sample is taken. For this reason the percentage error on the determination is always the same. All our reduction determinations *are done in quadruplicates* and the average value taken. A great number of controls have shown that the maximum error in the determination of the reduction value in this way, that is, by taking the average of four analyses, is 0.001 mg. of glucose. If only one determination is made the error will be greater. The procedure of Hagedorn and Norman Jensen is undoubtedly the most accurate micro method for the determination of the reduction value of glucose.

E. Dialysis.

It is previously mentioned that polarimetric determination of the rotation power of a glucose solution can be made with accuracy

only if the solution is *perfectly* clear. This point is very important, and even if one may judge a solution to be clear by simply looking at it, it may happen that it is not absolutely transparent and colorless when one looks through a layer 20 cm. long in the polarimetric tube. Only in spinal fluid and edema fluid can one expect such a transparency of the fluid drawn from the body that it can be used without special preparation. In the spinal fluid not the slightest amount of blood must be present. In cases of pleural fluid one may be able to clarify the fluid by simply cleansing it by means of charcoal. When we want to determine the rotation value of the sugar in the blood and of the sugar in our experiments *in vitro* (to be described in Paper VII) the solutions cannot be used directly, nor can they be clarified with charcoal. In such instances we use dialysis, as previously briefly described (1). The whole procedure of dialysis must be performed in such a way that: (1) A perfectly clear solution is obtained. (2) The solution does not contain protein. (3) The greatest possible amount of sugar comes through in the shortest possible time. This is dependent on two circumstances: (*a*) the character of the membrane, and (*b*) the construction of the dialysis apparatus.

Preparation of the Membrane.

We use Kahlbaum's membrane collodion always. The membranes are made by pouring collodion on the outside of a glass tube of definite size, and great care is taken that the glass tube is of uniform diameter throughout. Before the glass tube is used, it is cleansed with soap and water and perfectly dried. Then a rubber stopper is introduced in each end of the glass tube. The two rubber stoppers have holes exactly in the middle. Through these holes a glass rod is introduced and used as an axis for rotating the glass tube. During the rotation the glass tube is kept absolutely horizontal by inserting the free ends of the glass rod into two holders. When the collodion is poured on the glass tube, it is rotated by hand with a speed of about 20 rotations to a minute. The amount of collodion poured on is dependent on the size of the membrane and cannot be given in exact figures. It is poured on in three layers, and the first layer is *not* allowed to dry before the next layer is poured on. Care is taken that the collodion is distributed as evenly as possible on the surface. After the last

layer has been poured on, the glass tube is still rotated for 2 minutes. Then the ether of the collodion will be vaporized, and the glass tube with the collodion membrane is now put into a solution of 70 per cent alcohol, where it remains for 10 minutes. Then it is removed and introduced into a basin with running water. After having been in the water for about 10 minutes the membrane can be loosened from the glass tube. In doing that it is usually necessary to cut away the ends of the collodion layer, partly because the ends adhere firmly to the glass tube, partly because the collodion may be somewhat unevenly distributed at the ends.

TABLE VI.

Determinations of the thickness of three collodion membranes. 10 determinations are made on each membrane at different places.

Membrane 1.	Membrane 2.	Membrane 3.
mm.	*mm.*	*mm.*
0.37	0.36	0.43
0.40	0.36	0.39
0.43	0.39	0.42
0.35	0.40	0.45
0.43	0.37	0.41
0.40	0.38	0.40
0.42	0.36	0.42
0.45	0.35	0.44
0.39	0.36	0.43
0.41	0.36	0.42
Average..0.405	0.369	0.421

Through a series of preliminary investigations (7) we have found out that a membrane made in this way gives the best and fastest dialysis. If a more dilute alcohol solution is used, the protein may go through, and if a more concentrated solution of alcohol is used, the dialysis takes a longer time. It is mentioned above that we cannot state exactly the amount of collodion necessary for making membranes. According to our experiments the alcohol concentration is more important than the thickness of the collodion membrane for the rate of dialysis. This, however, holds true only if the thickness of the collodion membrane is kept within certain limits. This can be found only through experience. In

Table VI we have given the thickness of three such membranes. The determinations are made by cutting from a collodion tube two narrow pieces from one end to another. Then ten determinations of the thickness are made, five for each slip. Below the columns in Table VI is given the average thickness for the membranes. The deviation for the average value is rather small, and it is necessary to be very careful in the preparation of the membrane in order to obtain such a uniformity. As a general rule, therefore, we can state that the thickness of the membrane may not exceed $\frac{1}{2}$ mm.; if it should be too thin at any place the protein may go through. If the collodion membranes are not to be used immediately they are kept in distilled water to which a little toluene is added. Kept in this way, they remain constant for at least a week.

Construction of the Dialysis Apparatus.

The dialysis apparatus has the following construction (see Fig. 1): One end of the collodion tube is closed with a rubber stopper. The tube is fastened to the stopper by means of a band of strong rubber tube, and the connection must naturally be perfectly tight. The rubber stopper has in its center a hole for a glass rod. This glass rod is longer than the collodion tube and can be used as a handle when the tube has to be moved from one place to another. The collodion tube is introduced into a glass cylinder. The fluid to be dialyzed is then put into the collodion tube, whereas physiological salt solution is outside the collodion membrane. By varying the diameter of the cylinder the relation between the amount of the inner and outer fluid can be changed. When the inner fluid has been introduced into the collodion tube a rubber stopper of the same size as the one used at the end is pushed over the glass rod into the upper part of the collodion tube. This last stopper keeps the walls of the collodion tube in place, so that the glass rod always is in the middle of the collodion tube. Another somewhat larger rubber stopper which fits the cylinder can be pushed down over the glass rod, so that the distance from the membrane to the walls of the cylinder is kept constant. If the dialyzing apparatus is constructed in such a way that the surface of the membrane is about 2 sq. cm. to each cc. of the inner fluid a sufficient amount of sugar has dialyzed from the

blood in 1½ hours. By increasing the area for dialysis it takes still shorter time. If the size of the membrane is 3½ sq. cm. to each cc. of the inner fluid sufficient dialysis is obtained in ½ hour if the same membrane is used as before. The relation between the surface area of the dialyzing membrane and the amount of the inner fluid can be increased by using a glass tube within the membrane instead of a glass rod. In that case the inner

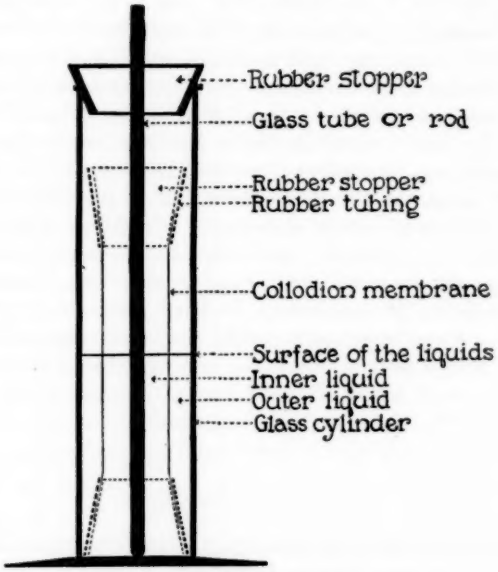

FIG. 1. Dialysis apparatus.

fluid lies like a relatively thin layer on the inner side of the membrane. The amount of salt solution used outside the membrane may vary. In most of our experiments the amount of inner fluid has been twice the amount of fluid outside the membrane. In such cases the glucose concentration in the dialysate after 1½ hours (or ½ hour) will be about one-half the concentration of the glucose in the inner fluid. The dialysis is not continued until equilibrium is obtained. In another publication in this Journal (7) we have reported our studies on the standardization of collodion membranes. To this the reader is referred for further information.

F. Procedure in Producing New-Glucose in Vitro.

In the first publications of this series we showed that it was possible to produce new-glucose *in vitro* by addition of insulin and muscle tissue to a solution of ordinary α,β-glucose. We shall describe the details of this procedure here.

Osmotic Condition and Reaction of the Glucose Solution.

The solution of α,β-glucose must be isotonic with the muscle tissue. This is the case with a glucose solution of 5.4 per cent. When a smaller concentration of glucose is used, the original 5.4 per cent solution is diluted with a 0.9 per cent solution of sodium chloride. In this way any (isotonic) glucose concentration below 5.4 per cent can be made. (If muscle tissue from cold blooded animals is used, the glucose solution must be isotonic with a 0.6 per cent solution of sodium chloride.) The glucose solution used must always be prepared some time before the experiment is started in order to secure equilibrium between the α- and β-fraction of ordinary glucose which in dried state consists of only α-glucose. The glucose and the sodium chloride must be dissolved in water with a neutral reaction. In order that the reaction of the solution shall not be changed during the experiment, buffers are added. The isotonic glucose solutions are always heated to 37°C. before the addition of insulin and muscle tissue.

Addition of Muscle Tissue.

The muscle tissue is taken from animals which are killed by decapitation without using narcotics. A certain amount of muscle tissue (15 gm. in most of our experiments) is taken out as fast as possible and cut in very small pieces. Then it is added to the glucose solution; the whole procedure of this part of the experiment takes not more than 10 minutes.

Insulin.

It is necessary to be very careful in selecting the insulin. In all our experiments we have used insulin specially prepared for us by Det danske Kemikalie- og Medicinal-Kompagni. Many of the usual insulin preparations contain sugar and therefore cannot

be used. The insulin must naturally not contain any substances which have either reduction power or rotate the polarized light, nor must it contain substances (for instance antiseptics) which might prevent the production of new-glucose or increase its mutarotation. In Paper I we have given certain figures as to the concentration of insulin necessary to secure the production of new-glucose. The units used in this publication are the so called Scandinavian units or Leo units which are about four times as strong as the units of most other countries. The figures given for the amount of insulin in Table V of Paper I (4) must therefore be *multiplied by four* if they are to correspond with the usual units. It is previously stated that buffers may be used in order to secure a constant neutral reaction of the solution. When buffers are added to a solution of insulin a precipitation may occur which may be heavy enough to inactivate the insulin. The degree of precipitation by buffers differs in the various insulin preparations, and it is always necessary to control this beforehand. If Sørensen's phosphate solutions are used as buffers the precipitation usually is very heavy in most insulin preparations. We have therefore in most cases used boric acid buffers, and only if the precipitation is very slight can insulin be used.

Shaking the Mixture.

The glucose solution to which muscle tissue and insulin are added is shaken thoroughly in a shaking machine rotating in a thermostat at 37°C. We have usually added either glass beads or metal pieces to the solution in order to secure a good mixture. The bottle used for mixing has a volume about three times as great as the volume of the solution used.

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STUDIES IN CARBOHYDRATE METABOLISM.

VII. INVESTIGATIONS INTO THE TRANSFORMATION OF THE LIVER GLYCOGEN INTO GLUCOSE IN VITRO.

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INTRODUCTION.

In an earlier paper[1] we have shown that by the simultaneous action of fresh muscle tissue and insulin at 37°C. on ordinary α,β-glucose, a form of glucose is produced which is characterized by a lower specific rotation value than that of α,β-glucose. This hitherto unknown form of glucose reverts to α,β-glucose in neutral reaction at 18°C. in the course of 48 hours. We have proposed for the present to call it "new-glucose."

In some subsequent papers[2] we investigated whether this form of glucose could be detected in the organism. We discovered that new-glucose was constantly present in the blood of normal persons, but that it could not be demonstrated in the majority of patients examined with diabetes mellitus. On the other hand it was always found in the blood of patients with diabetes mellitus 2 hours after a dose of insulin. Lastly we detected it in various body fluids of patients with normal carbohydrate metabolism. We concluded from these investigations that in the organism the α,β-glucose absorbed is changed into new-glucose by insulin and a substance present in the muscles. The further fate of new-glucose in the organism is not known but we must assume that its production is absolutely necessary for normal carbohydrate metabolism.

The reason that we now turn our attention to the question of the significance of the *liver* in carbohydrate metabolism is because

[1] Lundsgaard and Holbøll (1924–25).

[2] Lundsgaard and Holbøll (1925).

of the great rôle, judging from numerous physiological and clinical observations, which this organ is supposed to play in this process. The questions which are a natural consequence of our previous investigations, are, primarily, the following.

(*a*) Is new-glucose a necessary link in the formation and deposition of liver glycogen?

(*b*) What form of glucose is liver glycogen transformed into by glycogenolysis *in vivo* and *in vitro?*

(*c*) Is liver tissue, like muscle tissue, capable of forming new-glucose from α,β-glucose with the aid of insulin?

If, in dealing with the first question, we compare the knowledge we have gained through our earlier work of the action of insulin and the production and occurrence of new-glucose under different conditions, with the experimental and clinical experience of others concerning the action of insulin in the organism, it seems reasonable to assume, *at any rate as a provisional working hypothesis, that new-glucose constitutes an essential link in the formation of glycogen.* For this reason we have, for the present, not made this question the subject of an experimental investigation, although, as mentioned, it is the first problem which arises.

In this paper we shall give an account of our investigations into the second question, namely into what form of glucose the liver glycogen is transformed, but we have only investigated the post-mortem transformation of liver glycogen. The last of the three problems will be discussed in a separate paper.

Previous Investigations.

That glycogen is changed into glucose post mortem, and that this transformation is due to an enzyme, has been known ever since Claude Bernard's time. He supposed that the enzyme was also present in the liver cells *in vivo*, and took part in the formation of sugar *in vivo*. It was, however, not until much later through the work of Tebb, Wohlgemuth, Zegla, and Macleod and Pearce, that the existence of glycogen-splitting enzymes in the liver cells could be regarded as settled. Finally, in 1913, Bang showed by an investigation of the isolated, still living, frog liver preserved in Ringer's solution, that the formation of glucose in the liver is a vital process of the liver cells and can take place independently of the action of the nervous system and the internal secretion.

Since the discovery of insulin a number of workers have made investigations into the effect of insulin on the production of glycogen and sugar by the liver. Thus Macleod and Noble have carried out experiments with arti-

ficial circulation through the isolated liver of the tortoise. They perfused the liver with glucose solutions with and without insulin. The experiments showed that the glycogen formation was not increased when the perfusion fluid contained insulin. Lesser and his coworkers made similar experiments on frog liver with exactly the same result. They have further examined the effect of adding adrenalin to the perfusion fluid. Adrenalin causes an enormous increase in the yield of sugar by the liver. They found that this is just as pronounced whether the perfusion fluid contains insulin or not.

Authors' Investigations.

In order to determine what kind of glucose glycogen is transformed into post mortem, the specific rotation value of the glucose must be estimated as soon as possible after it has been liberated. The technique adopted was as follows: The animal employed for the experiment was the guinea pig which was killed by decapitation. Immediately afterwards the liver was taken out, weighed, quickly minced, and 20 gm. were put in a 300 cc. flask which contained 200 cc. of physiological salt solution heated to 37°C., to which were added 5 cc. of Sørensen's phosphate mixture with a pH of 7.30. The flask was then put in a shaking machine at 37°C. The reason that we chose to experiment at this reaction although Langfeldt has shown that glycogenolysis in sodium chloride solution has its optimum at pH 6.80, was because we wished to work at the reaction at which the decomposition of glycogen presumably takes place in the organism.

The amount of glucose formed in the experiments was estimated by determining the glucose content of the mixtures by Hagedorn and Norman Jensen's method. In order to estimate the specific rotation value of the glucose we had to compare the reduction and rotation values as before. The rotation value of the mixture could not be measured directly and we adopted therefore the same technique as described in our earlier works; namely, to determine the specific rotation value of the glucose in the dialysate after dialysis through collodion membranes. In ½, 1½, and 2½ hours time a sample of the mixture was withdrawn and put in a dialyzer. Dialysis took place at 20°C. Collodion membranes which had been immersed in 70 per cent alcohol were used in these experiments, as before. A thick glass tube was fitted into the interior of the collodion membrane whereby a large dialyzing surface in relation to the volume of fluid was obtained.

By this maneuver the diffusion of the glucose was considerably accelerated, as described in another paper,[3] so that a dialysis time of half an hour sufficed. As soon as the dialysis was finished the rotation value was read. This was determined as the mean of 40 readings. The reduction value of the glucose in the dialysate was determined as the mean of four analyses by the potassium ferricyanide method.

TABLE I.

Amount of Glucose Liberated from 20 Gm. of Liver at 37°C. in Physiological Salt Solution at pH 7.30.

Time after experiment was started.	Amount of glucose liberated.					
	Experiment I.	Experiment II.	Experiment III.	Experiment IV.	Experiment V.	Experiment VI.
hrs.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
1	0.481	0.476	0.389	0.550	0.498	0.341
2	0.725	0.676	0.592	0.802	0.748	0.483
3	0.780	0.750	0.645	0.893	0.817	0.500
4	0.806	0.773	0.655	0.936	0.820	0.500
5	0.813		0.664			0.506

TABLE II.

Specific Rotation Value of Glucose in the Dialysate of Experiments in Table I.

Time after experiment was started.	Specific rotation value.					
	Experiment I.	Experiment II.	Experiment III.	Experiment IV.	Experiment V.	Experiment VI.
hrs.						
½	+53.3°	+51.2°	+52.5°	+53.7°		+51.2°
1½	+51.9°	+51.8°		+51.3°	+53.9°	+52.5°
2½	+51.7°	+52.3°	+52.3°	+51.6°	+51.4°	

Six experiments were made. The results are given in Tables I and II.

In Table I will be found the determinations of the total amount of glucose split off. One of the experiments is also graphically represented in Fig. 1. The reason that more glucose was not liberated in 4 to 5 hours was not because all of the glycogen present in the liver was decomposed, but merely that a state of equilibrium corresponding to the given conditions had been established.

[3] Lundsgaard, C., and Holbøll, S. A., *J. Biol. Chem.*, lxviii, 439.

As is seen from the table the amount of glucose varies in the individual experiments. This was only to be expected as the glycogen content of the livers used must certainly have been different to begin with.

In Table II the values of the specific rotation of the glucose in the dialysate in six experiments are recorded. It will be seen that the rotation values agree, within the experimental error, with the rotation value of α,β-glucose: $+52.5°$.

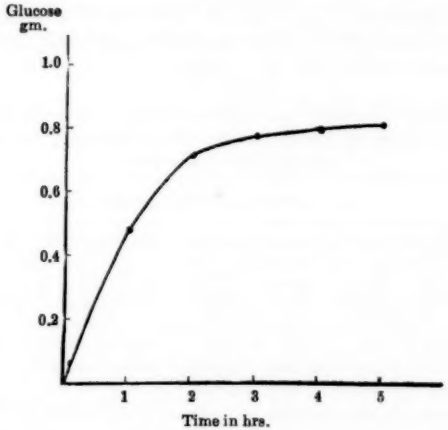

FIG. 1. The amount of glucose liberated from 20 gm. of liver at 37°C. in physiological salt solution with a pH value of 7.30. Experiment I in Table I.

With this series of experiments we have thus succeeded in showing *that the liver glycogen, immediately after the animal's death, preserved in physiological salt solution at a temperature of 37°C. and at a reaction of pH 7.30, was split into* α,β*-glucose.*

Does Insulin Influence the Decomposition of Glycogen in Vitro?

The next point we investigated was whether the presence of insulin altered the course of the glycogenolysis quantitatively

or qualitatively. After Langfeldt's demonstration that glycogenolysis is increased quantitatively by the addition of adrenalin and thyroiodine, it was interesting to investigate the effect of insulin on the process from this point of view. The second question which we intended to clear up was whether the action of insulin on the glucose formed from the glycogen of the liver cells could cause its transformation into new-glucose. The experiment was conducted in the following manner. The liver of a large guinea pig was carefully minced and two exactly equal portions of 15 gm. each were weighed out. Each of these portions was then put in a 300 cc. flask at 37°C. As in the previous ex-

TABLE III.

Amount of Glucose Liberated from Liver Glycogen in Four Series of Experiments. In Each Series, One Experiment Was Made with the Addition of Insulin and One without.

Time after experiment was started.	Amount of glucose liberated.							
	Experiment I.		Experiment II.		Experiment III.		Experiment IV.	
	Without insulin.	With 5 units of insulin.	Without insulin.	With 10 units of insulin.	Without insulin.	With 20 units of insulin.	Without insulin.	With 50 units of insulin.
hrs.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
1	0.324	0.330	0.246	0.235	0.463	0.460	0.362	0.375
2	0.502	0.500	0.362	0.350	0.702	0.695	0.547	0.570
3	0.540	0.536	0.391	0.379	0.745	0.750	0.600	0.618
4	0.546	0.541			0.770	0.779	0.612	0.629

periments, the flasks contained 200 cc. of physiological salt solution and 5 cc. of phosphate mixture with a pH value of 7.30. To one of the solutions a definite quantity of an insulin solution of known strength was added. The insulin solution possessed neither reduction power nor the ability to rotate polarized light. The flasks were next put in a shaking machine at 37°C. Samples of the mixtures were taken for the determination of the total amount of glucose liberated, and also samples of the mixtures containing insulin, after intervals of $\frac{1}{2}$, 1, and $1\frac{1}{2}$ hours for the estimation of the specific rotation value after dialysis through collodion membranes, the procedure being otherwise the same as previously described.

In Table III the amount of glucose liberated in four experiments is given. The only factor that was varied was the quantity of insulin, which was 5, 10, 20, and 50 units respectively in the four experiments. The results show that at corresponding times the amount of glucose liberated was the same in the two flasks with and without insulin. This proves that glycogenolysis is quantitatively quite unaffected by the addition of insulin.

In Table IV the values of the specific rotation angle of the glucose in the dialysate from the samples removed from the mixtures containing insulin are given. It appears from the values found that the glucose occurs in the form of α,β-glucose. From this series of experiments we can draw the conclusion

TABLE IV.

Specific Rotation Value of Glucose Liberated from the Liver in the Presence of Insulin.

Time after experiment was started.	Specific rotation value.			
	Experiment I.	Experiment II.	Experiment III.	Experiment IV.
hrs.				
½	+52.5°	+54.0°	+51.9°	+53.8°
1½	+51.6°	+51.0°	+52.5°	+52.3°
2½	+53.6°	+52.2°		+50.9°

that insulin has no influence on the postmortem decomposition of glycogen, either quantitatively or qualitatively.

The amount of glucose liberated in the mixtures containing insulin was exactly the same as in the quite analogous experiments without insulin, and the glucose formed in both cases was α,β-glucose.

DISCUSSION.

We have discovered by our investigation that in the freshly removed liver tissue, at 37°C. in physiological salt solution and at the normal reaction of the organism, glycogenolysis proceeds by the transformation of the glycogen into α,β-glucose. As we made the experimental conditions as near as possible to the conditions *in vivo* we have good reason to believe that it is also changed into α,β-glucose *in vivo*. This is important for the understanding of certain clinical findings associated with pathological

carbohydrate metabolism. Petrén has shown that an increase of blood sugar may take place in the later part of a fasting period. Faber and Iversen have seen that in diabetic patients treated with insulin the blood sugar concentration practically always rises in the night, so that it is comparatively high early in the morning. As the cause of this rise Faber imagines that insulin produces an accumulation in the depots, which yield up the sugar to the blood again in the night. Hatlehol has further investigated this nocturnal rise and has shown that it occurs not only in diabetics treated with insulin but also in those on a limited diet without insulin.

If our assumption that glycogen *in vivo* is given up in the form of α,β-glucose is correct, the nocturnal rise in the blood sugar concentration is intelligible. Under the influence of insulin part of the carbohydrates will be burned and part accumulated in the form of glycogen. Later on, when insulin is no longer present in the blood, the glycogen, for reasons which are still obscure, will again be split up, the product being α,β-glucose, which can only be burned (or deposited again) by the further influence of insulin.

This shows the great difficulty of substituting one or two daily subcutaneous injections of insulin for the internal secretion of the pancreas. The practical lesson to be learned from this fact in the treatment of diabetics with insulin is to strive to adjust the dose of insulin to the carbohydrates in the food as accurately as possible. It also seems to indicate that it is a disadvantage to a diabetic to get his glycogen depots full during insulin treatment, because the insulin deposits some of the glucose absorbed as glycogen which is again decomposed into α,β-glucose and thus needs a further supply of insulin.

For a more complete understanding of the part played by the liver in carbohydrate metabolism, it is, however, essential to investigate the third question we mentioned in the beginning of this work; namely, whether liver tissue in conjunction with insulin can form new-glucose from α,β-glucose.

SUMMARY.

1. It has been shown that the glycogen in freshly removed, minced liver tissue kept at 37°C. in physiological salt solution with a reaction of pH 7.30, is transformed into α,β-glucose.

2. This process is influenced neither quantitatively nor qualitatively by the addition of insulin.

3. The results obtained seem to explain the nocturnal rise in the blood sugar concentration in diabetics, which has been observed clinically.

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STUDIES ON CARBOHYDRATE METABOLISM.

VIII. INVESTIGATIONS INTO THE ACTION OF LIVER TISSUE AND INSULIN ON GLUCOSE IN VITRO.

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INTRODUCTION.

In a previous article[1] we have shown that the liver glycogen is decomposed into α,β-glucose *in vitro* under experimental conditions which correspond to those present in the glycogenolytic processes *in vivo*. We further found that the addition of insulin had no effect upon the course of the glycogenolysis, either as regards the amount or the kind of glucose which was split off. These results seem to afford an explanation of the clinical observation that the blood sugar concentration in diabetic patients often rises greatly during the night.

To gain more complete knowledge of the part played by the liver in carbohydrate metabolism it is necessary to investigate whether liver tissue, like muscle tissue, is able in association with insulin to form new-glucose from α,β-glucose. We were now able to investigate this problem since in the preceding work we found that the liver glycogen was split off as α,β-glucose. The production of new-glucose in this way is, therefore, excluded, which might otherwise have affected the results.

Of earlier work in this field there exist only a few experiments of Winter and Smith in which they investigated the effect of adding both liver extract and insulin to glucose and fructose. In the first case they found a decrease and in the second case an increase in the rotation angle.

[1] Paper VI (Lundsgaard and Holbøll, 1926).

EXPERIMENTAL.

The method employed was as follows: The freshly removed and finely divided liver tissue was put in a 300 cc. flask, and 200 cc. of a solution containing a definite quantity of α,β-glucose solution, physiological salt solution, and 5 cc. of phosphate mixture with a pH value of 7.30 were added. Finally insulin was introduced. The flask was placed in a shaking machine at 37°C. The different factors were varied in a series of experiments. The amount of liver tissue varied from 10 to 50 gm. The glucose concentration in the mixtures varied from 0.8 to 5.0 per cent. The insulin solution added contained between 2 and 50 units. Lastly the duration of the experiments varied between $\frac{1}{2}$ and 24 hours. The determination of the specific rotation angle was made by removing a portion of the mixture and putting it in a dialysis apparatus. After half an hour's dialysis the outer liquid was removed, which was then water-clear and suitable for a polarization determination. The rotation value was determined as the mean of 20 readings. The reduction value was determined—if necessary after dilution—by the potassium ferricyanide method. In Table I all the experiments are recorded. The experimental conditions and the results will be seen in the table. The specific rotation value was found to lie between $+50.7°$ and $+54.0°$, so that no formation of new-glucose was demonstrated. The experiments show us *that fresh liver tissue, unlike fresh muscle tissue, is unable in the presence of insulin to form new-glucose from* α,β*-glucose.*

Winter and Smith have found, as already stated, that the addition of liver extract and insulin to glucose causes a decrease in the specific rotation angle. It is difficult to understand that liver extract can exert an action which cannot be detected by using freshly minced liver instead. Winter and Smith's published experiments, however, were only accessible to us in such an abbreviated form that further discussion must be deferred until more information is obtainable.

Eadie, in Macleod's laboratory, has repeated Winter and Smith's experiments with liver extract. He was, however, unable to confirm the experiments.

In some of the experiments in this series the glucose concentration in the original mixture was determined during the whole

TABLE I.

Specific Rotation Value of Glucose after Being Acted Upon by Insulin and Fresh Muscle Tissue.

Insulin units.	Liver.	Glucose.	Duration of experiment.	Specific rotation value.
	gm.	*per cent*	*hrs.*	
50	50	2.0	½	+52.1°
50	50	2.0	2	+52.8°
50	50	2.0	6	+52.7°
50	50	2.0	24	+52.0°
50	10	1.0	½	+51.2°
50	10	1.0	1	+51.0°
50	10	1.0	2	+52.3°
20	10	0.8	1	+52.2°
20	10	0.8	2	+51.9°
20	10	0.8	3	+52.0°
20	50	3.2	½	+51.0°
20	50	3.2	1	+50.7°
20	50	3.2	12	+52.0°
20	50	3.2	24	+51.6°
10	20	5.0	½	+54.0°
10	20	5.0	1	+51.5°
10	20	5.0	2	+51.2°
10	20	5.0	3	+52.4°
10	20	0.8	½	+52.0°
10	20	0.8	1	+53.7°
10	20	0.8	2	+52.3°
5	20	1.0	½	+51.0°
5	20	1.0	1	+51.0°
5	20	1.0	2	+52.4°
5	20	1.0	24	+51.8°
2	20	1.2	½	+51.5°
2	20	1.2	1	+52.5°
2	20	1.2	2	+52.2°
2	20	1.2	3	+51.6°
2	20	1.2	4	+53.8°

experiment. It was found that it increased. This rise was due, of course, to a decomposition of the glycogen. In a few of the

experiments equal quantities of the same liver were added to a mixture without glucose and to a mixture with glucose. In both mixtures the glucose concentration was determined at suitable intervals. The increase in the glucose was the same in the two cases, which shows that the amount of glucose formed from the glycogen was the same in both cases. The experiments show that the original amount of α,β-glucose was not broken down and did not disappear during the experiment.

Simultaneous Action of Muscle Tissue, Liver Tissue, and Insulin on Glucose.

Finally we have investigated what effect the addition of liver tissue has upon the new-glucose formed by muscle tissue and insulin. The experiment was carried out in the following manner: To a solution, heated to 37°C., of 230 cc. of a 1 per cent α,β-glucose solution in physiological salt solution containing phosphate mixture, of a pH of 7.30, were added 20 units of insulin and 15 gm. of fresh, finely divided muscle tissue. After 2 hours action at 37°C. a sample of 30 cc. was removed for the determination of the specific rotation angle of the glucose. The remainder was divided into two equal portions, both of which were kept continuously at 37°C. To one portion were added 15 gm. of fresh, finely divided guinea pig liver. 15 gm. of the same liver were added to 100 cc. of physiological salt solution at 37°C. with a pH of 7.30. The glucose concentration and the specific rotation value for the glucose were determined in all the three flasks at intervals for some hours.

In Table II the glucose concentrations found in the three flasks are recorded. Flask A is the one which contained only muscle tissue and insulin, Flask B, the corresponding one to which liver tissue was added after 2 hours action, and Flask C contained liver tissue and physiological salt solution at a pH of 7.30. The results show that while the glucose concentration in the first flask remained unchanged during the experiment, it increased, as expected, in the two other flasks owing to the splitting up of the liver glycogen.

If we now compare the values for the glucose concentration found in Flasks B and C we find that the increase is the same in the two containers. This proves that the glycogenolysis has

proceeded unchanged in spite of the addition of muscle tissue, and that the new-glucose formed in Experiment B (compare Table III) has not undergone any breaking down or transformation from the addition of liver tissue. In Table III the results of the determinations of the specific rotation angle for the glucose in the samples taken during the course of the experiment are recorded.

TABLE II.

Glucose Concentration in the Three Mixtures.

A, glucose + muscle tissue + insulin.
B, " + " " + " + liver tissue.
C, liver tissue.

Time after main experiment was started.	Glucose.		
	Experiment A.	Experiment B.	Experiment C.
hrs.	*per cent*	*per cent*	*per cent*
2	1.000	1.000	0
3	1.010	1.392	0.386
4	1.004	1.557	0.562
5	0.993	1.612	0.628
6	0.990	1.629	0.634

TABLE III.

Specific Rotation Value for Glucose in the Three Mixtures.

A, glucose + muscle tissue + insulin.
B, " + " " + " + liver tissue.
C, liver tissue.

Time after main experiment was started.	Specific rotation value.		
	Experiment A.	Experiment B.	Experiment C.
hrs.			
2	+42.5°	+42.5°	
4	+43.3°	+46.4°	+52.6°
6	+43.8°	+47.2°	+52.6°

In Mixture A, 2 hours after the muscle tissue and insulin were added, we find the specific rotation value to be +42.5°, which shows that the formation of new-glucose demonstrated in our earlier work has taken place. The values after 4 and 6 hours are a little higher, indicating that the mutarotation has begun. If we look at the results of Experiment B, in which

liver tissue was added after the first 2 hours had elapsed, we find that the specific rotation value is decidedly greater than the corresponding samples in Experiment A, which shows that the concentration of new-glucose compared with the concentration of α,β-glucose is smaller in Experiment B than in Experiment A. At first sight this might perhaps be construed as indicating that there was a decrease in the absolute amount of new-glucose in Experiment B, either on account of an increased mutarotation or from a breaking down or transformation of the new-glucose by the aid of the liver tissue. This last view, however, was negatived by the results of the glucose concentrations. There is, however, another explanation, the amount of glucose is increased through decomposition of the glycogen by

TABLE IV.

Found and Calculated Specific Rotation Values for the Glucose in Experiment B from Tables II and III.

Time after main experiment was started.	Specific rotation value.	
	Found.	Calculated.
hrs.		
4	+46.4°	+46.1°
6	+47.2°	+47.1°

the addition of liver tissue. The liberated glucose is α,β-glucose; this follows from the results reported previously and from the values of the specific rotation in Experiment C. The increase in the amount of α,β-glucose will manifest itself in an increase in the specific rotation value, although the absolute amount of new-glucose is kept constant. From the results in Tables II and III of Experiments A and C, we can calculate what value we must expect to find for the glucose in Experiment B. The found and calculated results are entered in Table IV. The absolute agreement proves the correctness of the explanation we have given of the increase in the specific rotation angle in Experiment B.

In this experiment, as mentioned, the liver tissue was not added until 2 hours after the muscle tissue and insulin had begun to act. In this way it was ensured, as we knew from earlier experiments, that the muscle tissue had lost its power of forming new-

glucose and, therefore, could not exert any effect on the α,β-glucose split off from the liver glycogen. In order to prove that muscle tissue in conjunction with insulin is able to form new-glucose from the α,β-glucose split off from the liver glycogen, the following experiment was made. To 200 cc. of physiological salt solution at 37°C. and pH 7.3, 30 gm. of liver tissue and 20 units of insulin were added. After 4 hours action at 37°C. a sample was removed for the determination of the specific rotation angle. The result found was +52.8°. Next, 15 gm. of fresh, finely divided muscle tissue were added and after 2 hours action a new sample was removed which gave the specific rotation value +39.3°.

The experiment shows *that while insulin alone has no influence on the decomposition of the glycogen, since, just as is usual in vitro, α,β-glucose will be formed, the introduction of muscle tissue causes the transformation into new-glucose.*

The experiment shows decisively the importance of muscle tissue for the action of insulin.

DISCUSSION.

Our investigations show that liver tissue, unlike muscle tissue, does not possess the power, in conjunction with insulin, of forming new-glucose. Although according to our previous investigations, the production of new-glucose must be attributed to vital cell activity of the muscle tissue, this property may not be present in all cells. Whether the production of new-glucose, which occurs as the result of the action of muscle tissue and insulin on α,β-glucose *in vitro*, is due to a specific power connected with the muscle tissue cannot yet be settled, as we have not yet excluded the possibility that cells from other organs than the liver have the same capacity as muscle cells for producing new-glucose. That the negative results of the experiments with liver tissue might be due to the liver tissue exercising an inhibiting effect on the action of the insulin, is excluded by the results of the experiments where muscle tissue and liver tissue were employed at the same time.

If we compare this result with the fact brought to light in our preceding work, that the liver glycogen is broken down into α,β-glucose, we can conclude that the part played by the liver in carbo-

hydrate metabolism is that the organ serves as a depot for glucose in the form of glycogen, while it has no direct bearing on the combustion of glucose in the organism. This is in agreement with recent important physiological experiments of Burn and Dale. They worked with decapitated cats from which all the viscera were removed, so that they obtained a preparation consisting of the heart, vessels, and the skeletal muscles. In such preparations they showed that insulin produced a characteristic blood sugar fall and that much more sugar was got rid of under the action of insulin than without insulin. Burn and Dale's experiments show that the initial step in the combustion processes which occur in the presence of insulin—the disappearance of the glucose from the circulation—can take place in an absolutely normal manner even though all the tissues except the heart and skeletal muscles are absent. One of the conclusions they draw is the following: "It [is] impossible to suppose that an essential step in the disappearance of dextrose is its conversion by the liver, under the influence of insulin, into a reactive form." Neither Burn and Dale's results nor our own exclude the possibility that other organs than muscle tissue and pancreas are involved in the carbohydrate metabolism, as it takes place in the intact organism, but the experiments, at any rate, do not allow us to ascribe to other organs than the muscle tissue and pancreas, a definite, to say nothing of an indispensable rôle in the combustion of sugar.

Some Theoretical Remarks about the Course of Carbohydrate Metabolism in Normal Persons and Diabetics.

In conclusion we will attempt to review the transformation processes which we assume that the glucose molecule undergoes in its decomposition in the organism. In Fig. 1 we have, purely diagrammatically, put forward the conception of carbohydrate metabolism which we have arrived at by comparing previous experiences and hypotheses with the results we have obtained in our investigations. Ordinary α,β-glucose, denoted by the first large circle, comes mainly from the carbohydrates of the food; only a small portion is derived from the protein bodies. Now we know that the first transformation of the glucose molecule is from α,β-glucose into new-glucose. In our first investigation

we have shown that this process, outside the organism, is set in action by insulin and an unknown substance derived from muscle tissue. There can hardly be any doubt that the same two sub-

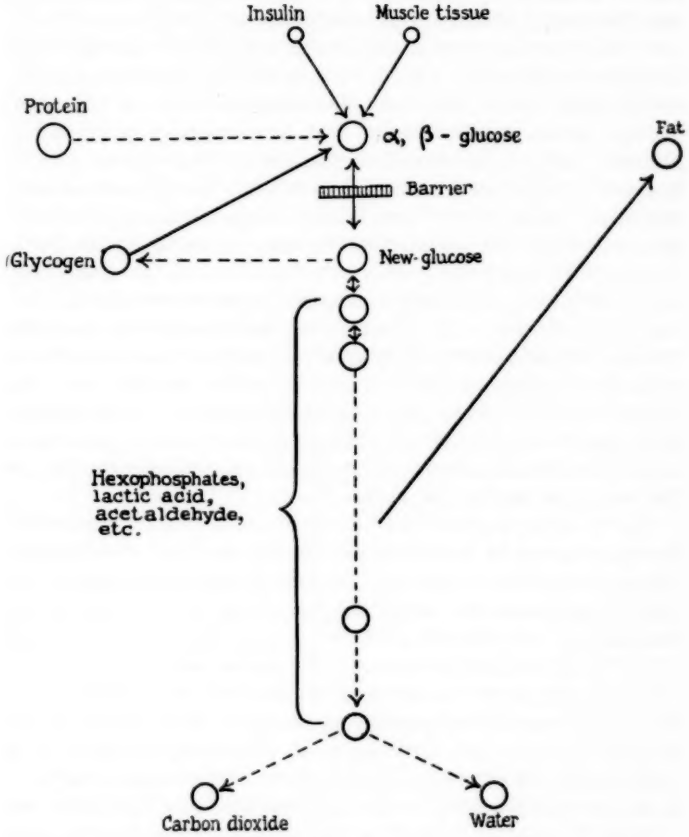

FIG. 1.

stances—insulin and the substance from muscle tissue—are necessary for the transformation in the organism. *In vitro* the process is reversible, new-glucose being slowly reconverted into α,β-

glucose. In the organism the fate of new-glucose is, however, certainly different, since, unlike what happens *in vitro*, there is an opportunity for a further transformation (breaking down or polymerization into glycogen) of this substance so that any mutarotation is thereby counteracted or prevented.

In the figure an arrow leads from new-glucose to the circle for glycogen; in this way we have tried to indicate that new-glucose can be deposited in the form of glycogen. This has not been directly shown experimentally, but can be put forward as a probable working hypothesis on the basis of a number of clinical and experimental facts. It must for the present remain doubtful whether new-glucose is *directly* transformed into glycogen, or whether the transformation takes place by one or more intermediate stages and possibly through some of the connecting links which form the direct connection between new-glucose and the end-products, carbonic acid and water. In Fig. 1, from practical considerations, we have let the glycogen formation proceed directly from the circle which stands for new-glucose. An arrow leads from the glycogen up to α,β-glucose. In our earlier work we have in fact shown that glycogen is broken down into α,β-glucose, which can only be transformed into new-glucose by the action of insulin and muscle tissue.

Besides being transformed into glycogen we must assume that new-glucose can be broken down directly, and that after passing through a number of more or less well demonstrated connecting links (hexophosphates, lactic acid, acetaldehyde, etc.) it is finally decomposed into the end-products CO_2 and H_2O. It is probable that the first of these processes is reversible, which we have indicated in the figure by the use of double arrows. From one (or more) of these unknown connecting links the synthesis of the fat originating in the carbohydrates in all probability proceeds as is indicated by an arrow. This points in one direction only, as there is no evidence that carbohydrates can be formed from fat.

While this sketch must be assumed to correspond to the conditions relating to carbohydrate metabolism in the normal organism, they are different when the carbohydrate metabolism is pathological. In our investigations into the form of glucose present in the blood of normal persons and patients suffering from diabetes mellitus, we found, like Winter and Smith, that

new-glucose could always be detected in the blood of normal persons but only rarely in diabetics. In diabetics the blood sugar is entirely or partly present in the form of α,β-glucose. We must, therefore, conclude that the metabolic anomaly in diabetes occurs at the first transformation step of glucose in the organism; namely, from α,β-glucose to new-glucose. At this point there is a barrier or blockade of the normal transformation processes. In the diagrammatic figure we have indicated this barrier by a horizontal bar. We must assume that the barrier, which manifests itself by a diminution or cessation of the production of new-glucose, is due to the decreased or absent production of insulin in diabetics, whereas an absence of the action originating in the muscle tissue has not yet been demonstrated as the cause of diabetes.

Although we cannot at present make all the symptoms in diabetics dependent—directly or indirectly—upon such a barrier, yet there is no convincing fact which proves that other anomalies are *primarily* concerned in the carbohydrate metabolism in diabetes mellitus. It seems, therefore, not to be unreasonable to advance, as a provisional working hypothesis, the theory *that the pathogenesis of diabetes mellitus must be entirely due to an obstruction in the course of the transformation of* α,β*-glucose into new-glucose.*

The action of insulin in diabetics is then easily understood. The insulin introduced, in conjunction with the active body in muscle tissue, will transform α,β-glucose into new-glucose and thus do away with the barrier. The insulin (and the muscle tissue) *start* the metabolism of glucose in the organism, for since insulin helps in the formation of new-glucose, it prepares the way for the subsequent transformation processes, the normal course of which is so essential to the organism.

We have previously mentioned that we believe that the formation of new-glucose is absolutely necessary for the transformation of glucose in the organism. As may be seen from the diagram new-glucose occupies a specially important position in the course of the transformation processes. From this point[2] the transformation processes can proceed in two directions, for new-glucose may either be accumulated in the form of glycogen, or

[2] Compare, however, the discussion relating to the formation of glycogen.

burned. What these relative proportions of new-glucose in any individual case are, in other words, how much is deposited as glycogen and how much is burned, must be dependent on a number of factors.

Under normal conditions it does not matter whether new-glucose is burned or deposited as glycogen, because when the glycogen is again split up into glucose there will always be a sufficient production of insulin in normal persons to transform the α,β-glucose again into the form necessary for the organism, namely new-glucose. In diabetics the conditions are different. In them the amount of new-glucose deposited as glycogen will, so to speak, be lost to the metabolism when the glycogen returns in a cyclic manner to α,β-glucose, a form which the diabetic organism cannot utilize. The amount of new-glucose which is accumulated as glycogen and the corresponding amount of insulin do therefore not contribute to the immediate supply of energy to the organism. If the decomposition of glycogen into α,β-glucose occurs at a time when there is no insulin present, a rise in the blood sugar takes place, possibly to such a height that some of the glucose is excreted by the kidneys. This part of the insulin action must, therefore, be looked upon as lost. As previously mentioned, the transformation of glycogen into α,β-glucose explains the clinically observed nocturnal rise in the blood sugar concentration in diabetics.

SUMMARY.

1. It has been shown that liver tissue, unlike muscle tissue, cannot form new-glucose from α,β-glucose in conjunction with insulin.

2. It has been shown that a solution containing new-glucose does not undergo any change on the addition of liver tissue. No formation of glycogen can be demonstrated *in vitro*.

3. From this and our preceding work we can draw the conclusion that the part played by the liver in carbohydrate metabolism is to serve as an accumulation depot for glucose in the form of glycogen, while it has no direct effect on the combustion of glucose in the organism.

4. Lastly, a diagrammatic representation of the initial transformation processes of glucose in the organism is given.

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THE SOLUBILITY OF VITAMIN B IN BENZENE.

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That vitamin B is soluble in water or dilute alcohol is a matter of general agreement. It has also been reported repeatedly that the substance is soluble in dilute acetone or dilute methyl alcohol. With regard to its solubility in absolute alcohol, there is some disagreement, but there is no evidence of more than a sparing solubility in this solvent except in the presence of mineral acid. In view of the ease with which absolute alcohol becomes contaminated with moisture, no reported evidence can be regarded as conclusive that there is any appreciable solubility in absolute alcohol. Certainly all experience shows that considerable dilution of alcohol with water aids the solution of vitamin B. The same is true of acetone. It was therefore surprising to learn from the work of McCollum and Simmonds[1] that the substance, under certain special conditions, is soluble in so highly immiscible a solvent as benzene. Such a peculiar property, if established in a thoroughly trustworthy manner, should be of great use as a means of separating the vitamin from foreign matter. We have therefore examined the special conditions described by McCollum and Simmonds and offer below what appears to be an explanation of their results.

They found that benzene does not extract the vitamin from white beans or wheat embryo, but that if the raw material is extracted with 95 per cent alcohol and the alcoholic extract is evaporated on dextrin, extraction with benzene in a continuous type of extractor will remove the vitamin from the dextrin. The products were tested by the use of rats.

McCollum and Kennedy[2] had previously reported two cures of

[1] McCollum, E. V., and Simmonds, N., *J. Biol. Chem.*, 1918, xxxiii, 55.
[2] McCollum, E. V., and Kennedy, C., *J. Biol. Chem.*, 1916, xxiv, 491.

polyneuritis in pigeons by a benzene extract of the original foodstuff. No record was published of any changes in weight of the birds, nor was the duration of life stated. It is to be doubted that these authors now attach much weight to these two curative results under the circumstances. The distinction drawn by Mitchell[3] between vitamin B and the antineuritic substance receives very slender support from this particular bit of evidence, though his general contention may well be correct.

We have twice paralleled the experiment of McCollum and Simmonds, using rice polish as our raw material and pigeons as experimental animals in preventive tests. Benzene does not extract the protective substance directly from rice polish but does dissolve the vitamin if the residue of the alcoholic extract is repeatedly treated with the hot hydrocarbon in a continuous type of extractor. So far we find ourselves in complete harmony with McCollum and Simmonds. However, if the protective substance is first adsorbed on fullers' earth and afterward liberated therefrom by alkali or otherwise, a solution is obtained which yields a dry residue from which benzene will not extract any active substance. If such a vitamin preparation is suspended in 95 per cent alcohol and the alcohol is then evaporated, the protective substance is still not extractable by benzene. In order to render the substance in this form soluble in benzene, we tried the addition to the benzene of fats, oleic acid, lecithin, and cholesterol, with the idea that such substances accompanying the vitamin in the alcoholic extract of rice polish might influence its solubility in benzene. We also made an alcoholic extract of rice polish, removed the vitamin therefrom with fullers' earth, and after careful removal of the alcohol used the residue in conjunction with benzene in an attempt to dissolve vitamin previously liberated from fullers' earth. We also attempted to esterify the vitamin by reaction with various alcohols and to convert it into an acetal by the use of ortho-formic ester. In no case could we dissolve the protective substance in benzene.

We then repeated our previous experiment of extracting rice polish with 95 per cent alcohol, and after evaporating off the bulk of the alcohol, noted that a faint alcoholic odor persisted in the residue, even after long warming on the steam bath. We there-

[3] Mitchell, H. H., *J. Biol. Chem.*, 1919, xl, 399.

fore dried the residue at 60° *in vacuo* for several hours, and then subjected it to overnight extraction in a Soxhlet apparatus with benzene. The benzene failed to extract the vitamin, which was demonstrated by feeding test to be present still in the extracted residue.

The explanation appears to be a very simple one; *viz.*, that pure benzene never dissolves the vitamin but does remove it from alcoholic extracts which still retain small amounts of water and alcohol, as alcoholic extracts of foodstuffs are very prone to do. It seems safe to assume that the effective solvent is the low boiling ternary mixture of alcohol 18.5 per cent, water 7.4 per cent, and benzene 74.1 per cent, which distills off at 64.85°C. from a mixture[4] of any proportions. One can readily imagine how, in a continuous type of extractor, any small amount of alcohol and water present would be utilized again and again independently of the much larger amount of benzene present.

We verified our conclusion in a measure by extracting the vitamin readily with benzene containing 10 per cent of 95 per cent alcohol from material which had previously been removed from fullers' earth and from which prolonged extraction with pure benzene had failed to remove the vitamin. Addition of 10 per cent absolute alcohol to the benzene did not produce the same effect as an equal proportion of 95 per cent alcohol. We also saturated rice polish with 90 per cent alcohol and allowed the polish to dry in the air. We were then able to extract the active substance directly from the rice polish with benzene, since the air-dried rice polish still retained some alcohol as well as water.

However, the low boiling mixture of alcohol, water, and benzene is by no means highly selective and we have concluded that there is little promise in the use of immiscible solvents for the separation of vitamin B.

Some slight question still remains as regards the relation between the results of McCollum and Simmonds and our own, due to our use of different raw material and different experimental animals. We have as yet been unable to repeat their experiments more precisely.

[4] Barbaudy, J., *Compt. rend. Acad.*, 1925, clxxx, 1924.

THE COMPARATIVE METABOLISM OF CERTAIN AROMATIC ACIDS.

X. FATE OF *m*-NITRO-, *m*-AMINO-, *m*-HYDROXY-, AND *m*-CHLOROPHENYLACETIC ACIDS IN MAN, THE DOG, AND THE RABBIT.

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Phenylacetic acid itself (1) does not combine with glycocoll in the human organism, as does benzoic acid. Its derivatives investigated up to the present time have been excreted by man in the urine either free or combined with glycine instead of glutamine. Of the compounds tested in this form of experimental work, the nitro (2), amino, and hydroxy (3) derivatives, as well as several of the halogen (4) derivatives of both the ortho and para series of phenylacetic acids, none has been detoxicated by a glutamine conjugation. Hoping for an explanation of this unique reaction by possibly duplicating it, we studied the metabolism of *m*-nitrophenylacetic acid, *m*-aminophenylacetic acid, *m*-hydroxyphenylacetic acid, and *m*-chlorophenylacetic acid.

EXPERIMENTAL.

The substances were ingested as water solutions of their sodium salts or fed in this form by means of a stomach tube to dogs and rabbits. The urine was collected for 36 to 48 hours after the last dose and evaporated to a thick syrup on a water bath, cooled, acidified to Congo red with sulfuric or phosphoric acid, and then extracted with some organic solvent in a rotary extracting apparatus.

m-Nitrophenylacetic Acid.

Preparation.

m-Nitrobenzyl chloride in alcoholic solution was treated with a saturated water solution of potassium cyanide, according to the

usual method, and the mixture boiled under a reflux condenser for 5 hours. The alcohol was then distilled off and the *m*-nitrobenzyl cyanide was extracted from the potassium chloride with absolute ether. The cyanide as purified by distillation *in vacuo*. It distils over as a pale yellow liquid at 175–180°, under 13 mm. pressure; melting point, 63°.

The *m*-nitrobenzyl cyanide was converted into *m*-nitrophenylacetic acid by hydrolysis with hydrochloric acid. After several recrystallizations from water, a product melting at 122° was obtained.

m-Nitrophenylacetyl Chloride.

This compound was made according to the usual procedure in making acid chlorides with thionyl chloride. It is very unstable at high temperatures, but may be distilled at 66° under 7 mm. pressure. The acid chloride is a crystalline yellow solid, melting at 77°.

m-Nitrophenylacetic Ethyl Ester.

This compound, prepared in the usual way from the acid chloride and ethyl alcohol, is an unstable oily liquid, boiling at 39° under 12 mm. pressure.

Metabolism of m-Nitrophenylacetic Acid.

A man weighing 80 kilos ingested 8 gm. of *m*-nitrophenylacetic acid in a period of 26 hours as a water solution of the sodium salt. No ill effects were noted. The urine, collected for 36 hours after the final dose, amounted to 3600 cc. This was evaporated, acidified to Congo red with sulfuric acid, then repeatedly extracted with ether. A total of 4.1 gm. of uncombined *m*-nitrophenylacetic acid was extracted from the urine. No conjugation product could be identified.

A dog of 25 kilos was fed 8 gm. of the acid in four 2 gm. doses, and was kept on a spare diet for 48 hours while the urine was collected. This was extracted with ethyl acetate and the extract evaporated to dryness. The residue was dissolved in hot water and two fractions of crystals obtained. The first fraction to appear, after repeated recrystallizations, melted at 120°. It proved to be unchanged *m*-nitrophenylacetic acid. This uncombined portion amounted to only 0.5 gm., or 6 per cent. The more

soluble fraction, when purified, melted at 175°; in appearance it resembled phenaceturic acid. The substance proved to be a glycocoll conjugate of *m*-nitrophenylacetic acid, as it possessed the same properties as the synthetic compound. Hydrolysis by acid yielded *m*-nitrophenylacetic acid and glycocoll. Titration results are as follows:

0.1362 gm. substance required 5.90 cc. 0.1 N NaOH; calculated 5.72 cc.
0.1454 " " " 6.30 " 0.1 " " " 6.11 "

Nitrogen determinations, according to the method of Dumas, gave the following results.

0.0921 gm. substance gave 6.65 cc. at 19°, 756 mm.
0.1261 " " " 9.10 " " 18.5, 756 "
Calculated for $C_{10}H_{10}N_2O_5$. N 11.76.
Found. " 11.75.
" 11.90.

This fraction amounted to 1.9 gm., or 24 per cent of the amount fed. A gummy residue, which refused to crystallize, remained. It appeared to be a urea additive compound of *m*-nitrophenaceturic acid as solution of the gummy mass in barium hydroxide and subsequent treatment with dilute sulfuric acid split off urea and allowed *m*-nitrophenaceturic acid to crystallize. From this we obtained 0.7 gm., or a total of 33 per cent.

A rabbit was fed 4 gm. of *m*-nitrophenylacetic acid in 1 gm. doses on each of 4 consecutive days. 1.93 gm. (48 per cent) of the substance were recovered from the urine as unchanged acid. No conjugation product could be found.

Synthesis of m-Nitrophenaceturic Acid.

5 gm. of *m*-nitrophenylacetyl chloride, m.p. 77°, were dissolved in 35 cc. of chloroform and gradually added with continuous shaking to a slightly alkaline water solution of 3.5 gm. of glycocoll. A few drops of dilute sodium hydroxide were added from time to time, in order to keep the reacting mass slightly alkaline to litmus. After all the acid chloride had been added, the contents of the shaking funnel was strongly acidified to Congo red with hydrochloric acid, setting free *m*-nitrophenylacetic acid. After filtering, the filtrate was extracted with ether, to recover

the dissolved *m*-nitrophenylacetic acid. The filtrate was then evaporated at room temperature by means of an electric fan. The *m*-nitrophenaceturic acid (*m*-nitrophenylacetyl glycocoll) crystallized out in small white irregular crystals. It is much more soluble in water than is the *m*-nitrophenylacetic acid; in fact, its solubility is much the same as that of phenaceturic acid. The new compound is quite insoluble in ether and acetone, but soluble in ethyl acetate and alcohol. After several recrystallizations from water and drying at 85° *in vacuo*, it melted at 176°.

Analyses.

0.1410 gm. substance gave 0.2606 gm. CO_2 and 0.0521 gm. H_2O.
0.1211 " " " 0.2240 " " " 0.0457 " "
0.1062 " " " 7.65 cc. N at 19°, 758 mm.

Calculated. C 50.42, H 4.10, N 11.76.
Found. " 50.42, " 4.29, " 11.84.
" 50.45, " 4.14.

m-Aminophenylacetic Acid.

Preparation.

This compound was prepared by the reduction of *m*-nitrophenylacetic acid according to the method of Jacobs and Heidelberger (5). The yield varied between 25 to 30 per cent of the theoretical. The acid is considerably more soluble than the *p*-aminophenylacetic acid and shows a much greater tendency to turn red on standing. It was recrystallized twice from water and when dried melted at 147–149°.

Metabolism.

A man ingested two doses of 2 gm. each of *m*-aminophenylacetic acid during a 48 hour period. The urine was extracted with ether and ethyl acetate. The evaporated urine was extracted with chloroform, benzene, and petroleum ether. The extracts were then evaporated and taken up in hot water. The water solution, made 40 per cent acid by the addition of sulfuric acid, was then boiled under a reflux condenser for 2 hours. Only the merest traces of *m*-aminophenylacetic acid could be found after the hydrolysis. A total of 1.6 gm. (40 per cent of the amount taken) was recovered from the first ethyl acetate extraction. No glutamine or other conjugate of *m*-aminophenylacetic acid could be found in the urine.

4 gm. of *m*-aminophenylacetic acid were fed to a dog of medium size, as a solution of the sodium salt, by means of a stomach tube; two 2 gm. doses were given during a period of 48 hours. In determining its excretion, the same procedure was followed as in our analysis of the human urine. Little or no conjugated acid was indicated by hydrolysis. From the 4 gm. fed, we recovered 1.5 gm. of the uncombined *m*-aminophenylacetic acid, or 38 per cent of the initial amount.

Absence of reducing substance in the urine of the human being and the dog would exclude the probability of a glycuronic acid complex.

Two rabbits received 1 gm. each on 4 consecutive days, making a total dosage of 8 gm. All the rabbit urines were united, evaporated, acidified, and extracted with ethyl acetate. This extract yielded two substances, one the uncombined *m*-aminophenylacetic acid, and the other a substance melting at 118–124°. This material was hydrolyzed by boiling alkali into *m*-aminophenylacetic acid and acetic acid. Analysis for nitrogen, according to Kjeldahl, gave the following results.

0.1314 gm. substance required 7.00 cc. 0.1 N acid.
0.1660 " " " 8.40 " 0.1 " "

Calculated for $C_{10}H_{11}NO_3$. N 7.25.
Found. " 7.46.
" 7.08.

Synthesis of m-Acetylaminophenylacetic Acid.

2 gm. of *m*-aminophenylacetic acid were heated with 10 gm. of acetic anhydride under a reflux condenser for 20 minutes. The reaction mixture was then poured into hot water and the *m*-acetylaminophenylacetic acid allowed to separate. From concentrated water solution, the substance crystallizes as thin yellowish leaves. It is very soluble in water and is gradually hydrolyzed by boiling water. Dried *in vacuo* at 60°, the substance melts at 122–124°. Nitrogen analysis, according to Kjeldahl, gave the following results.

0.2212 gm. substance required 11.50 cc. 0.1 N acid.
0.2010 " " " 10.50 " 0.1 " "

Calculated. N 7.25.
Found. " 7.27.
" 7.31.

m-Hydroxyphenylacetic Acid.

Preparation.

This substance was prepared according to the method of Salkowski (6) by the diazotization of *m*-aminophenylacetic acid. The yield is extremely poor, only 3 to 10 per cent of the theoretical. The melting point of the substance used was 128°.

Metabolism.

2 gm. of *m*-hydroxyphenylacetic acid were ingested by a man in a single dose. Only the free acid, and less than 1 gm. of it, was isolated from the urine.

2 gm. were fed to a medium sized dog, and approximately 1 gm. was recovered unchanged from the urine.

A rabbit was fed 1 gm. of the acid, and again only the free acid was found in the rabbit urine. The acid is entirely non-toxic and is rapidly eliminated in the urine. By means of Millon's reaction it is possible to follow qualitatively the time of excretion. After the feeding of 1 gm. to a rabbit, the substance can be detected in the rabbit urine after 20 to 30 minutes, and it appears that excretion is complete at the end of 3 to 4 hours, as the acid can no longer be identified in the urine by this delicate test.

m-Chlorophenylacetic Acid.

Preparation.

This compound was prepared according to the method of Sandmeyer from *m*-aminophenylacetic acid.

Metabolism.

A man weighing 67 kilos ingested 6 gm. of *m*-chlorophenylacetic acid. The urine was treated as described in previous experiments. About 2 gm. of the unchanged *m*-chlorophenylacetic acid were recovered from the urine and also 1.8 gm. of *p*-chlorophenaceturic acid but there was no trace of a glutamine compound.

6 gm. of *m*-chlorophenylacetic acid were fed to a dog. 4.8 gm. of *m*-chlorophenaceturic, m.p. 139–143°, were recovered from the urine, but none of the free acid could be found.

4 gm. of this substance were fed to a rabbit in 1 gm. doses on each of 4 consecutive days. Only a trace of free *m*-chlorophenylacetic acid could be isolated from the urine, but 3.77 gm. of white crystalline substance, melting at 141–143°, were extracted from the urine with ether. This substance was soluble in alcohol, ethyl acetate, methyl alcohol, and hot water, but insoluble in cold water, chloroform, and benzene. The substance on acid hydrolysis yielded *m*-chlorophenylacetic acid and glycocoll.

Analyses.

0.1873 gm. substance gave 0.3620 gm. CO_2 and 0.0758 gm. H_2O.
0.1419 " " " 7.60 cc. N at 19°, 755 mm.
Calculated for $C_{10}H_{10}O_3NCl$. C 52.63, H 4.38, N 6.11.
Found. " 52.71, " 4.50, " 6.12.

Nitrogen analysis, according to Kjeldahl, gave the following results.

0.2140 gm. substance required 9.40 cc. 0.1 N acid.
0.1826 " " " 7.75 " 0.1 " "
Calculated for $C_{10}H_{10}O_3NCl$. N 6.11.
Found. " 6.10.
" 5.94.

Synthesis of m-Chlorophenaceturic Acid.

m-Chlorophenylacetyl Chloride.

10 gm. of *m*-chlorophenylacetic acid were treated with 25 gm. of thionyl chloride, and the *m*-chlorophenylacetyl chloride distilled off at 52° under reduced pressure (16 mm.). The acid chloride is a yellowish green liquid. Analysis for chlorine, according to Carius, gave Cl 38.01 per cent. Calculated for $C_8H_6OCl_2$, 37.56 per cent.

m-Chlorophenaceturic Acid.

6 gm. of *m*-chlorophenylacetyl chloride were added drop by drop to a slightly alkaline water solution containing 5 gm. of glycocoll and sufficient 0.1 N sodium hydroxide solution to keep the reaction alkaline throughout the procedure. The contents of the flask was then made acid to Congo red with hydrochloric acid, filtered, and the filtrate extracted three times with ether to remove the *m*-chlorophenylacetic acid. The entire water solution, amounting to 250 cc., was evaporated by means of an electric

fan to 150 cc., when the *m*-chlorophenaceturic acid crystallized out in short white needles. After two crystallizations from hot water and drying at 70° *in vacuo*, the substance melted at 144–145°. Titration results are as follows:

0.1026 gm. substance required 4.50 cc. 0.1 N alkali. Calculated, 4.41 cc.
0.1208 gm. substance required 5.25 cc. 0.1 N alkali. Calculated, 5.31 cc.

Nitrogen analysis, according to Kjeldahl, gave the following results.

0.2140 gm. substance required 9.40 cc. 0.1 N acid.
0.1826 " " " 7.75 " 0.1 " "
Calculated for $C_{10}H_{10}NO_3Cl$ N 6.11.
Found. " 6.10.
" 5.94.

SUMMARY AND CONCLUSION.

m-Nitrophenylacetic acid passed unchanged through the body of man and rabbit but was conjugated with glycocoll in the body of the dog. In a previous publication (2) it was shown that *p*-nitrophenylacetic acid behaved in a similar manner when fed to these different subjects. On the contrary, *o*-nitrophenylacetic acid is eliminated unchanged in each of the three cases.

m-Aminophenylacetic acid behaves like *o*-aminophenylacetic acid (7); it is not conjugated by the human being or the dog but is acetylated by the rabbit. In contrast, *p*-aminophenylacetic acid, as previously shown (4), is acetylated by both the human being and the rabbit, being excreted as *m*-acetylaminophenylacetic acid, but is excreted in the urine of the dog as *p*-aminophenaceturic acid (a glycocoll conjugate).

m-Hydroxyphenylacetic acid was excreted unchanged by the human being, dog, and rabbit. This is true also of *o*-hydroxyphenylacetic acid (7). The *p*-hydroxyphenylacetic acid is excreted free (1) in the urine of man, dog, and rabbit, but by the monkey (3) is to a very small extent conjugated with glycocoll.

m-Chlorophenylacetic acid is in large measure combined with glycocoll by the human being, dog, and rabbit. *o*-Chlorophenylacetic acid and also *p*-chlorophenylacetic acid combine with

glycocoll in the human being and the dog but are excreted unchanged by the rabbit (4).

At present no satisfactory physical or chemical explanation can be offered for the detoxication of phenylacetic acid in the human body by a combination with glutamine, while with one exception all its derivatives so far studied either pass through the human body unchanged or are combined with glycocoll through the carboxyl group of the series of phenylacetic acids. It is notable that in the *p*-amino derivative acetic acid is found in combination with the amino group, forming a *p*-acetylaminophenylacetic acid.

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THE RELATION OF SOLUBILITY TO THE ABSORPTION OF CALCIUM SALTS FROM THE INTESTINE.

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Some earlier experiments (Irving and Ferguson, 1925) on the absorption of calcium chloride from the intestine of anesthetized dogs suggested acidity as an important factor in determining the velocity of calcium absorption. An aqueous solution of calcium chloride would not be affected in solubility within ordinary limits of hydrogen ion concentration, but it is presumable that the intestinal contents are quite different from a pure water solution. The concentration of dissolved calcium is probably a determining factor in the absorption of calcium. The experiments which are here reported indicate that this absorption is influenced strongly by the solubility conditions prevalent in the intestine.

Defective absorption is suggested by Findlay (1924) as the prime cause of the calcium deficiency usually found in rickets. Many investigators have inferred a connection between calcium deficiency disorders and the reaction and other solubility conditions of the diet. On a varied diet, however, it is difficult to predict solubility conditions in the absorbing organs, and rather impracticable to demonstrate them in survival experiments. Metabolism experiments, furthermore, do not show the degree of absorption of calcium because 90 per cent of its total excretion may occur through the feces (Salvesen, 1924). Consequently, the experimental conditions may favor excretion even to the point of exceeding the total intake, and no clear evidence on absorption itself is afforded.

EXPERIMENTAL.

The experiments were carried out with dogs under ether anesthesia and under similar operative conditions. Through an inci-

sion in the abdominal wall the calcium solutions of various salts with or without buffers were injected directly into the upper part of the intestine. The intestine had been previously tied off immediately below the pylorus. Under these conditions animals survived up to 7 hours. The conditions are abnormal but comparable, and the concentration of salts and buffers used was sufficient to give decisive results, but not so severe as to be immediately fatal.

Blood samples of about 15 ml. were drawn directly from the heart at the beginning and subsequently at half hour periods after the calcium salt injection had been made. Each blood sample was allowed to stand until the serum separated. The serum was decanted and centrifuged. Calcium was determined in these samples by Clark and Collip's (1925) modification of Tisdall's (1923) method.

Blood counts made in these and previous experiments indicated that no considerable changes in red cell concentration had occurred. In five animals examined the number of red cells varied from 3 to 6 per cent during the experimental period. According to this criterion there was no change in serum concentration sufficient to affect the trend of results.

The following solutions were used:

1. Calcium lactate solution was prepared by the slow titration of 1 mol of freshly ignited calcium oxide with 2 mols of standardized lactic acid. Addition of a few grains of calcium oxide produced a light pink with phenolphthalein. The calcium was so prepared because of the difficulty experienced in dissolving commercial forms. 5 ml. of 0.2 M Ca per kilo were injected.

2. The same calcium lactate as used in Experiment 1 was titrated with lactic acid to pH 3.5 with methyl orange. 5 ml. of solution per kilo were injected.

3. Weighed, freshly ignited calcium oxide was titrated to approximate neutrality with lactic acid and diluted to 0.3 M. The pH, determined electrometrically, was 6.58 at 25°. 20 ml. per kilo were injected.

4. A 0.2 M solution of calcium chloride was prepared and 20 ml. per kilo injected.

5. A solution of 0.2 M of $CaCl_2$ was prepared, 0.15 M sodium acetate added, titrated to pH 3.8 with acetic acid, and diluted to 1 liter. 20 ml. per kilo were injected.

6. 0.2 M calcium chloride, 0.05 M sodium citrate, and 1/60 M citric acid were dissolved, titrated with hydrochloric acid to pH

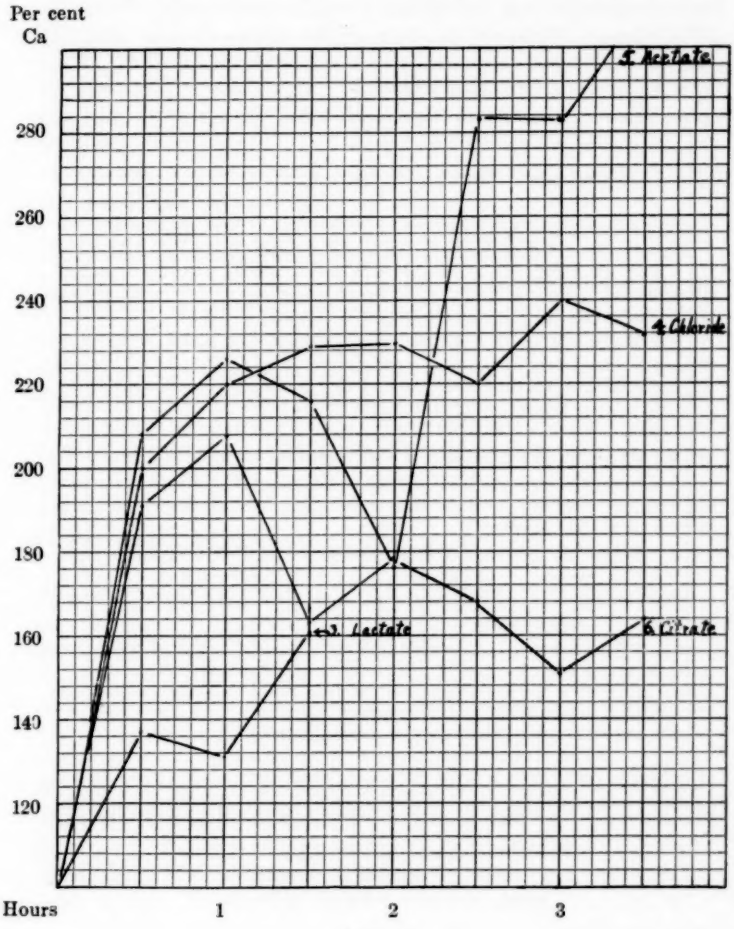

FIG. 1.

3.3, and diluted to 1 liter. 20 ml. per kilo were injected. Table I indicates the results of the experiments.

DISCUSSION.

Experiments 1 and 2 show the absorption of calcium lactate from an alkaline and acid lactate solution respectively. There is no consistent difference favoring either solution. The amount

TABLE I.

Serum Calcium Changes with Various Calcium Salt Solutions in the Intestine.

Experiment No.	Solutions.	Time, hrs.									
		0	½	1	1½	2	2½	3	3½	4	4½
1	0.2 M lactate, pH 8.5*	10.2	12.6	13.1	10.9	15.8	13.5	13.3	12.8	11.8	11.7
	5 ml. per kilo†	100	124	129	107	155	132	130	125	116	115
2	0.2 M lactate, pH 3.5*	12.8	15.2	18.0	17.9	16.1	16.7	15.6			
	5 ml. per kilo†	100	119	140	140	126	130	122			
3	0.3 M lactate, pH 6.58*	10.7	14.6	14.0	17.2						
	20 ml. per kilo†	100	137.	131	161						
4	0.2 M $CaCl_2$, pH 7.0*	14.2	28.4	31.4	32.5	32.7	31.3	34.0	33.0		
	20 ml. per kilo†	100	200	220	229	230	220	240	232		
5	0.2 M $CaCl_2$ acetate, pH 3.8*	12.5	26.0	28.2	27.0	22.0	35.4	35.4	39.2		
	20 ml. per kilo†	100	208	226	216	176	283	283	314		
6	0.2 M $CaCl_2$ citrate, pH 3.3*	10.6	20.2	22.0	17.3	18.9	17.9	16.0	17.4		
	20 ml. per kilo†	100	191	208	163	178	168	151	164		

* Calcium in mg. per 100 ml. of serum.

† Per cent Ca, taking first calcium content as 100 per cent.

of calcium presented would, if totally absorbed, elevate the serum calcium concentration to about 900 per cent of its initial value, for

5 ml. 0.2 M Ca = 40 mg. Ca.
1 kilo body weight = 50 ml. plasma.
50 ml. plasma = 5 mg. Ca (normal).

No such increase was observed. The greatest apparent increase (about 50 per cent) would correspond to the absorption of only about 2.5 mg. of Ca per kilo, or one-sixteenth of the amount avail-

able. From the fluctuations shown, however, it appears that both elimination and absorption are at work together, so that the calcium level at any time is a product of these two factors. The later stages show a decline in serum calcium, as if a limit had been reached beyond which excretion exceeded absorption. It has been found that the elimination of intravenously injected calcium is very rapid, requiring only 4 hours in dogs for 82.5 mg. of $CaCl_2$ per kilo to be totally eliminated from the serum (Bowler and Walters, 1924, p. 546). The same investigators found 386 mg. of $CaCl_2$ per kilo to constitute a lethal dose, so the doses used here are not of violent character.

Calcium lactate is found effective in restoring the serum calcium under certain deficiency conditions. Salvesen (1924, p. 62) notes its effect of increasing the serum calcium of parathyroidectomized dogs and warding off tetany symptoms. Inouye (1924) observed the relief of tetany in parathyroidectomized dogs on a lactose- or galactose-containing diet. He also comments on the acidophil character of intestinal flora favored by these diets, a condition which might be expected to favor absorption (Irving and Ferguson, 1925). Professor F. P. Underhill has remarked to me that recent experiments in his laboratory connect the favorable influence of a lactose diet for parathyroidectomized dogs with an actual increase in the blood calcium.

Calcium lactate is soluble to the extent of about 10 per cent, or 0.34 M. In ordinary commercial forms it dissolves very slowly, and electrometric titrations of calcium oxide with lactic acid were so slow in reaching equilibrium conditions that curves and solubility data were not obtained. It may also be that the amount of acid used for pH 3.5 reduced solubility instead of favoring it.

Experiments 3 to 6, using four times as much solution, show considerably more absorption. As 20 ml. of 0.2 M Ca contain 160 mg. of Ca, the maximum absorption (indicated in Experiment 5) of 13 mg. per kilo of dog is about one-twelfth of the amount available. This represents a higher relative absorption than occurred from 5 ml. of lactate solutions, as well as a much greater absolute absorption (see p. 518).

Experiment 4 shows the rapid absorption of highly soluble neutral calcium chloride. These increases in serum calcium are quite parallel to those found previously (Irving and Ferguson,

1925) for calcium chloride solutions. The curve showing rate of change in velocity of absorption also agrees in showing a steep rise to a maximum followed by a level period. Buffered acid calcium chloride solutions caused a continuous increase in serum calcium, representable by a nearly straight line almost to the time of death.

Such a continuous increase in serum calcium appears from the record of Experiment 5 (acetate) rising to the highest absolute and relative serum calcium in these experiments. The solubility of the salt used has an apparent relation to absorption. Table II shows the solubility of the various calcium compounds present in the mixtures. Calcium chloride in a solution acidified with acetate buffers is much more readily absorbed than neutral calcium chloride.

TABLE II.

Solubility of Calcium Salts. Estimated for 25° from Olsen's (1922) Tables.

Salt.	Solubility.
	M
Calcium chloride. $CaCl_2 \cdot 6H_2O$	5.4
" acetate. $Ca(C_2H_3O_2)_2 \cdot H_2O$	2.3
" lactate. $Ca(C_3H_5O_3)_2 \cdot 5H_2O$	0.34
" citrate. $Ca_3(C_6H_5O_7)_2 \cdot 4H_2O$	0.00017

Calcium chloride is less rapidly absorbed from a solution buffered with citrates than from the neutral or acetate solutions. This slowness conforms with the small solubility of calcium citrate. The earlier experiments (Irving and Ferguson, 1925), which showed a more rapid absorption of calcium from acid calcium chloride solutions buffered with citrates, apparently failed to show any restricting influence of citrates. The calcium chloride solutions were, however, much more concentrated and used relatively less citrate as buffer.

Summarizing the results indicates the following order for absorbability of calcium combinations with various anions: acid acetate > neutral chloride > acid citrate > acid lactate.

Maly (1876) remarked that NaH_2PO_4 diffused more rapidly than Na_2HPO_4 from a neutral mixture, so that the two could be quantitatively separated by repeated dialysis. He suggested

that acid substances generally diffused more rapidly than alkaline. The addition of alkali to milk in infant feeding brought about an unfavorable calcium balance (Sato, 1918). Milk acidified with hydrochloric acid proved effective in reducing the severity of tetany attacks in infants (Scheer, 1922). Acid administered intravenously relieved tetany and sodium bicarbonate aggravated the condition in parathyroidectomized dogs (Wilson, Stearns, and Janney, 1915), and hydrochloric was more effective than lactic acid. These authors suggested an alkalosis as contributing to tetanic conditions. In an extensive study of conditions during tetany from parathyroid removal, Collip, Clark, and Scott (1925) found no early change in alkaline reserve, and Salvesen (1924) had not found any significant variation. Consequently it does not appear that an alkalosis detectable in the alkaline reserve can be related to failure of calcium absorption.

Substitution of calcium chloride for lactate in a rickets-producing diet was sufficient to relieve rats of rachitic symptoms (Zucker, Johnson, and Barnett, 1922), while sodium bicarbonate or an alkaline diet otherwise similar induced rickets. Hydrochloric acid or even ammonium chloride favored recovery. Among the conditions determining proper calcium metabolism acidity and specific ion effects are named as probable important factors (Orr, Holt, Wilkins, and Boone, 1924). The evidence of these authors points to facilitation of calcium absorption by diets which would be plainly acid and favorable to calcium solubility during digestion.

Acid diets were found to increase the proportion of calcium in the urine and diminish that in the feces (Bogert and Kirkpatrick, 1922). But diets which cause a net acid excess in metabolism are not necessarily strongly acid during digestion. Furthermore, the determination of all fecal calcium as a true excretion is not precisely correct, for the feces contain that portion of calcium which is excreted from the blood and another part which has never been absorbed. Experiments in which the fecal calcium was increased over normal in two-thirds of their cases on basic diet may be a matter of diminished absorption.

Professor Gustav Embden has offered to the author the interesting suggestion that the order: acid acetate > neutral chloride > acid citrate > acid lactate corresponds with the Hofmeister series, if it is considered that the chloride member is

different in not being acidified. It is quite to be expected that the various ions would influence absorption by their effect on membrane permeability, but these experiments are not adequate to distinguish positively such an influence. In considering the solubility question alone there is also the matter of the influence of one neutral salt upon the solubility of another.

It is naturally also incorrect to assume that the other members of the absorbing system, the intestinal wall and the blood, are like a dead membrane in a pure water solution. Among the artificially varied conditions of these experiments, however, and from the other work referred to it seems that the solubility conditions are of considerable importance in determining the absorbability and absorption of calcium.

CONCLUSIONS.

Results of these experiments are in accord with the idea that calcium absorption is greatly affected by solubility conditions in the intestine. Under comparable conditions absorption of calcium salts proceeds in this order: acid acetate > neutral chloride > acid citrate > acid lactate.

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STUDIES ON THE EFFECT OF TEMPERATURE ON THE CATALASE REACTION.

1. EFFECT OF DIFFERENT HYDROGEN PEROXIDE CONCENTRATIONS.

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(Received for publication, March 23, 1926.)

In a recent investigation of the catalase activity of the blood (1) it is stated that the outstanding difference between catalase and other enzymes is "its remarkable activity at lower temperatures." It is well known, of course, that enzymes generally act best within a temperature range of 35–50°C. The great activity of catalase at low temperature is, therefore, a very striking phenomenon which merits closer study. Does the increased activity at low temperatures indicate an anomaly in the behavior of catalase as compared to other enzymes? Does catalase possess an optimum at the lower end of the temperature scale? In the case of blood catalase this apparent anomaly may perhaps be easily explained, as was done by Bach and Zubkowa (2), on the basis of the digestion of the catalase by blood proteases whose activity increases as the temperature rises up to 47°C. It seemed desirable to study this problem, using for this purpose a catalase preparation rather than whole blood in order to avoid complicating factors. Our experiments were all made with a catalase extract which is obviously free from proteases since it retained its activity for over 15 months without any observable deterioration.

Method.

The experiments recorded in this series of papers have all been made with catalase extracted from beef kidney. The cortex of a large number of kidneys was first finely chopped, then ground with sand in a mortar, and

extracted with an equal weight of water saturated with chloroform. The extraction proceeded for several days with frequent shaking, after which the watery extract was filtered through muslin, then through fluted papers. A clear, brown solution was obtained which was then treated with one-fifth its volume of chloroform. This treatment caused a precipitation of a large amount of protein and of dissolved hemoglobin, but the activity of the extract was not appreciably altered. The material was finally filtered once more and kept in a dark place in a glass-stoppered bottle. A small quantity of chloroform was added as a preservative. We performed experiments with this preparation for a period of over 15 months and during this time no material loss of the original catalase strength was found.[1]

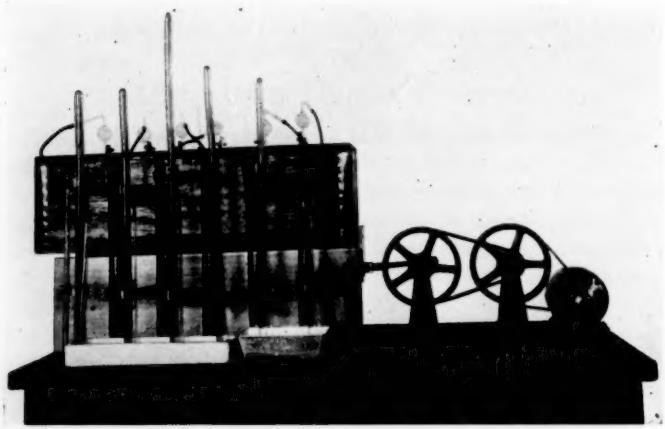

FIG. 1. Thermostat in which the catalase reaction takes place under constant shaking.

The different experimental temperatures were secured by means of a specially constructed thermostat shaking machine shown in Fig. 1.

The thermostat consists of a well insulated double walled box, the space between the inner and outer walls of which is lined with two layers of "insulatin," each 1 inch thick, separated by a 2 inch cushion of horse hair. The inside of the box is lined with zinc. The box is fastened to a shelf which slides freely in grooves and is attached by means of an arm to a set of wheels operated by an electrically driven motor. The entire thermostat with its contents is thus shaken at a fixed rate of about 100 strokes per minute.

Six determinations could be made simultaneously, the interior of the

[1]At the present time the preparation is more than 2 years old and still possesses its full strength.

box being provided with this number of holders for bottles in which the reaction took place. These are ordinary wide mouth glass bottles of about 500 cc. capacity, to which the various reagents were transferred directly. The catalase solution was measured into nickel crucibles, provided with a round knob on the bottom, which when lowered carefully into the bottle would remain in an upright position until the shaking began. The least jar sufficed to cause the crucibles to capsize and empty their contents into the bottle where the catalase solution immediately mixed with the other reagents.

The thermostat was filled with water of the desired temperature (or with ice) to such a depth that the six bottles were submerged up to the shoulder when placed in the holders. The latter were extensively perforated, thus permitting the water to flow freely about the bottles. The thermostat with the bottles in position was now closed, first by a zinc tray fitting snugly into the box but allowing the necks of the bottles to protrude freely. A layer of insulatin and a thick felt mat came on top of this and, finally, the thermostat was closed with a wooden cover. All these covers were so made that they fitted neatly around the neck of the bottles which alone remained exposed. The temperature of the water was determined by means of a thermometer inserted through the several covers to the bottom of the box.

The crucibles with the catalase solution having been lowered into the bottles, the latter were closed tightly with soft rubber stoppers carrying two tubes: one straight tube to which a piece of pressure tubing was attached and opened or closed by means of a screw clamp; and a bent tube provided with a bulb by which the evolved gas was conveyed from the reaction bottles into eudiometer tubes. The former served to keep the interior of the bottles at atmospheric pressure, allowing the free expansion or contraction of the air to take place during the preliminary period, while the temperature was equalized, without affecting the volume of the system. After the temperature of the entire system had been equalized, the bottles were closed off by tightening the screw clamps and the thermostat was now set in motion by turning the switch of the motor. The gas generated in the course of the reaction was collected over water in eudiometer tubes of different capacities according to the amount of oxygen expected in the particular experiment. The volumes of gas recorded in the tables have been corrected for temperature, barometric pressure, and aqueous tension, so that the data invariably represent volumes at standard conditions of 0°C. and 760 mm. of Hg.

In experiments of relatively short duration the temperature could be maintained generally within 1°C., though in experiments lasting several hours, except those at 0°C., a somewhat greater variation between the beginning and end temperature would take place. In this case, however, we could still secure a more or less definite temperature by leaving one reagent bottle vacant and packing ice into this to keep the temperature of the box from rising.

The total volume of the reaction mixture in our experiments was either 50 or 20 cc., the smaller volume being selected as our preference in all later experiments. The hydrogen ion concentration was also maintained con-

stant in all our experiments except where the variation in pH was the primary objective of the tests. This will be discussed in the last paper of this series, but it will be stated here that unless otherwise specified the reaction was always studied at a pH of 7.0, this being maintained with the aid of a phosphate buffer, the concentration of which in the total volume was 1/60 molar. We used Merck's Superoxol as the source of hydrogen peroxide. This is to be preferred to other grades because it is neutral and is not contaminated by various preservatives, it keeps well in a cool dark place, and the required concentration can be quickly prepared by appropriate dilution. We always checked up the peroxide content of the diluted material by direct titration with standard $KMnO_4$.

Effect of Temperature on the Catalase Reaction at Different Hydrogen Peroxide Concentrations.

We performed many experiments in which the catalase reaction was studied under varying hydrogen peroxide concentrations and temperature conditions. What impresses one at once in these experiments is the fact that at temperatures above 30°C. the reaction tends to become explosive, running a short but very vigorous course. At temperatures around 35°C. the reaction proceeds with great force and generally is completed in a minute or two. At temperatures of 30°C. or less the reaction velocity is very markedly affected, many hours being required for the reaction to run its full course below 10°C. As the velocity diminishes with a lowering of the temperature the extent of the reaction, as measured by the amount of oxygen set free, increases progressively up to a certain point, the maximum reaction, however, occurring above 0°C.

The marked difference in catalase activity under circumstances where temperature is the only variable factor may be due, of course, to the fact that its optimum, unlike that of other enzymes, is between 0 and 10°C. Issajew (3) claims that yeast catalase has an optimum of 40°C. but his experiments have not been properly controlled to determine the degree of spontaneous decomposition of the peroxide at high temperatures. In our experience this is so serious a factor that we could not rely on the results obtained at temperatures of 40° and over, and even experiments at 35–40°C. could be made only with the utmost precautions.

The observed difference in activity under different tempera-

tures, however, may also be due to other factors which either jointly or separately alter the catalase reaction. Thus, the loss of activity with rising temperature might be caused by a progressive spontaneous inactivation of the catalase, or it might result from an actual destruction of catalase by the excess of hydrogen peroxide. We shall attempt to analyze these factors with the view of gaining an insight into the correct interpretation of this peculiarity of the behavior of catalase at different temperatures. It has been already pointed out by one of us (4) that the catalase activity is depressed by increasing the hydrogen peroxide concentration. In fact, it is possible by rendering the concentration of the hydrogen peroxide sufficiently high to stop the reaction almost completely. This loss of activity is not a linear function of the peroxide concentration. In one experiment where we tested the effect of concentrations from 0.36 to 18.8 N, 99 per cent of the activity was lost through a 52-fold increase in the concentration; 76 per cent through a 13-fold increase, and 16 per cent through a 1.3-fold increase in the concentration. How does temperature affect the catalase reaction with varying hydrogen peroxide concentrations?

In Table I the results obtained in a number of experiments, wherein the combined influence of the two factors was studied, are summarized. It is quite obvious that the effect is cumulative, as is also illustrated by the curves in Fig. 2. The depressing effect of the hydrogen peroxide is intensified by a rise in temperature, but to varying degrees. From the series of curves in Fig. 2 it is seen that the greatest contrast in the catalase activity is manifested at 3° and the smallest at 24.5°C. In other experiments at still higher temperatures we succeeded in abolishing entirely the difference in activity with varying hydrogen peroxide concentrations. Working with a range of concentrations of 0.2, 0.3, 0.4, and 0.5 N 29 cc. of oxygen were given off at 34°C. in each instance, whereas at 0.5°C. 52, 64, 90, and 80 cc. of oxygen respectively were given off. The catalase concentration, of course, was the same in all experiments.

In the next paper it will be shown that spontaneous inactivation of catalase apparently does not take place under the conditions of our experiments. We are bound to conclude, therefore, that catalase must be destroyed by the excess of hydrogen

peroxide in the system. Thus there is in the catalase reaction an antagonism between the decomposition of hydrogen peroxide by the catalase with the resulting liberation of oxygen and the destruction of the catalase by the hydrogen peroxide which causes a diminution in the oxygen set free. The balance between

TABLE I.
Relative Catalase Activity.

Temperature.	H_2O_2 concentration.				
	0.36 N	0.54 N	0.72 N	0.90 N	1.08 N
°C.					
0	55.5	76.0	40.5	29.8	13.0
8		**100.0**	88.2	61.4	62.8
19.4	68.2	73.5	66.6	57.7	49.5
30	46.5	48.0	44.7	42.7	38.5
	0.36 N	0.72 N	1.08 N	1.44 N	1.80 N
3	**100.0**	82.6			49.0
11.5	95.5	70.9	53.0		
15.8	90.7		49.1	50.4	37.3
19.8	73.4	55.6	49.5	42.6	33.9
24.5	56.9	49.6	42.6	39.6	32.6
	0.37 N	0.55 N	0.74 N		
0		41.7	21.4		
10	**100.0**	82.5	56.0		
16.1	96.2	75.0	59.5		
20.2	71.5	60.5	52.4		
24.7	59.8	53.9	49.5		
29.5	49.0	44.5	41.2		
	0.22 N	0.36 N	0.50 N		
15.1		97.0	**100.0**		
20		78.0	70.0		
25.4	57.9	58.6	59.0		
30	48.2	44.8	44.8		

these opposed reactions is determined by temperature, on one hand, and by the relative peroxide concentration, on the other. At low temperatures the balance shifts in favor of the catalytic reaction, and at high temperatures, on the contrary, the destruction of the catalase by the peroxide is favored, leading to rapid

cessation of the catalytic reaction. The destruction of the catalase is presumably an oxidation phenomenon, and this would seem to be supported by the peculiarity of the temperature curves (Fig. 2). Greater catalytic activity in the cold is, therefore, due to less enzyme destruction through oxidation. At temperatures below 10°C. such destruction either does not occur

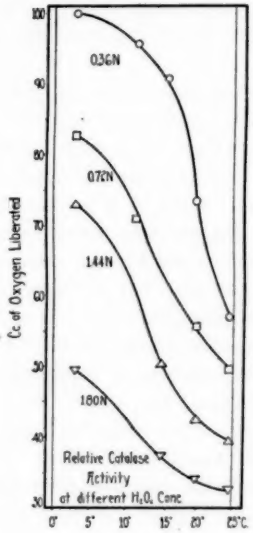

FIG. 2.

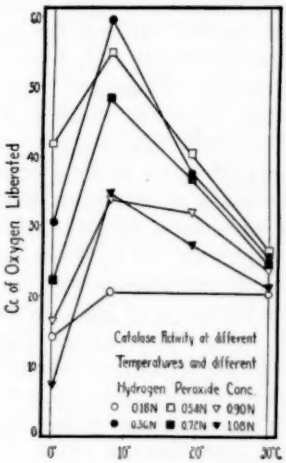
Cc of Oxygen Liberated
Catalase Activity at different Temperatures and different Hydrogen Peroxide Conc.
0.18N 0.54N 0.90N
0.36N 0.72N 1.08N
0° 10° 20° 30°C.

FIG. 3.

FIG. 2. Relative catalase activity at different H_2O_2 concentrations.

FIG. 3. Catalase activity at different temperatures and different hydrogen peroxide concentrations.

or else is very negligible; it is moderate between 10 and 20°C., but becomes much more extensive with further rise in the experimental temperature. At 35–40°C. the destruction is practically instantaneous in the presence of an excess of hydrogen peroxide. We are led to conclude, therefore, and this will find corroboration later on, that the rate of oxidation of the catalase increases more with temperature than does the catalytic reaction.

Although the catalase destruction below 10°C. no longer plays a significant rôle, the catalase reaction is definitely inhibited at 0°C. This is shown in Fig. 3, where the curves obtained at different hydrogen peroxide concentrations attain their maximum peak between 0 and 10°C. We maintain that it is erroneous to assume that the maximum activity occurs at 0°C. The reduced catalase activity at or near 0°C. is, of course, entirely different from the loss of activity sustained at high temperatures. As will be shown in the next paper, it is not due to inactivation of the enzyme by the low temperature.

A detailed study of the curves of the catalase reaction under different temperature conditions is very instructive, and some interesting things are brought out particularly in the initial stages, when the reaction is followed from minute to minute. At a definite hydrogen ion concentration (a pH of 7 in our experiments) and catalase concentration, the reaction commences practically at once upon mixing the reagents and there is usually an appreciable evolution of oxygen in the first few minutes no matter what the hydrogen peroxide concentration or the experimental temperature may be. At temperatures of 15 to 30°C. this liberation of gas continues until the reaction is completed. At lower temperatures, however, following the quick initial evolution of gas for a minute or two, the reaction stops for longer or shorter time, depending upon the temperature and the peroxide concentration. When again resumed, the reaction proceeds with interruptions which are longer the higher the hydrogen peroxide concentration and the lower the temperature. Thus, in one series of experiments at 3°C. and 0.36 N hydrogen peroxide the catalase reaction proceeded uninterruptedly and was completed in 190 minutes. At twice that concentration the reaction, after the first 2 minutes, remained inhibited for 15 minutes and, when it started up once more, only about one-fifth of the initial volume of gas was set free in the next 15 minutes. The reaction then proceeded slowly and was completed in 370 minutes. With still higher hydrogen peroxide concentrations (1.08 and 1.44 N) the immediate reaction was followed by a quiescent period of 25 minutes, while with 1.80 N, after the initial evolution of gas lasting 1 minute, the reaction remained in abeyance for 35 minutes, then it started up and continued uninterruptedly to the end.

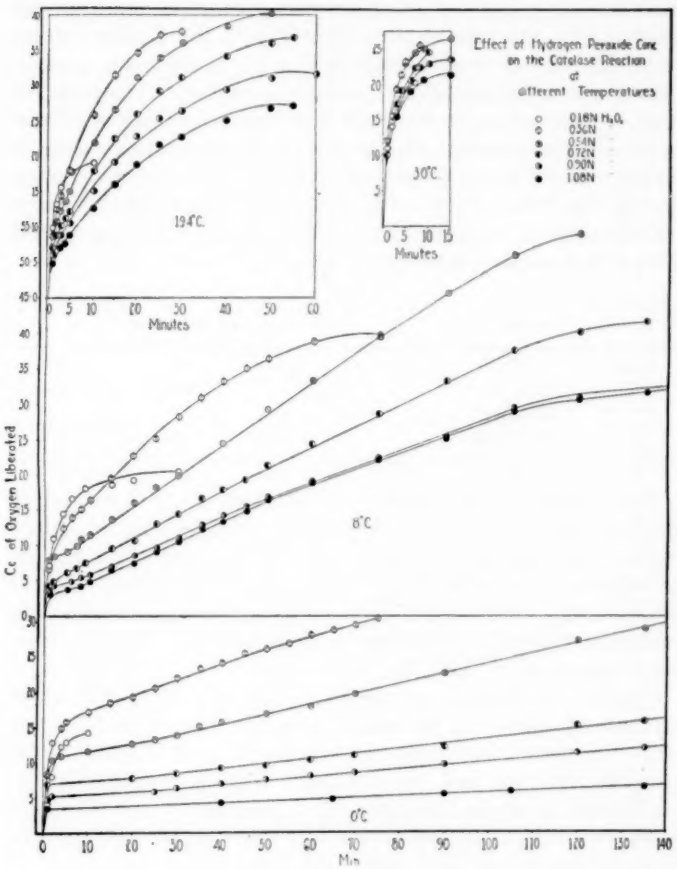

FIG. 4. Curves of four series of experiments with varying hydrogen peroxide concentrations and at different temperatures. The maximum activity has been found invariably with the 0.54 N H_2O_2. The curves at the lower temperatures are not completed, as the time at 0°C. extends to 270 minutes, but the direction of the curves is clearly indicated from the first half of the period. The curves at 8°C. with the greater peroxide concentration also extend beyond the limits of the figure, the reaction being completed after 195 minutes.

This condition has not been observed except at temperatures below 10°C. As can be seen from Fig. 4 the curves become markedly altered tending more and more to approach straight lines as the experimental temperature is lowered. The thing we wish to emphasize, however, is that even with high hydrogen peroxide concentrations and at very low temperatures there is almost invariably an immediate reaction upon mixing the reagents, the latent period of the reaction being relatively insignificant as may be shown, for instance, by the following records from two separate experimental series:

Temperature.	Latent period.
°C.	sec.
9.2- 9.8	10–15
21.0–21.4	8
31.1–31.4	0–3

Van't Hoff's temperature coefficient for a 10° temperature interval calculated from our experimental results is rather variable, though the variations are not haphazard but regular and depend upon both factors discussed in this section. There are obviously conditions of both temperature and peroxide concentration when the coefficient is quite constant. Some calculations are given in Table II.

The average temperature coefficient for this series is 4, which corresponds closely to the value found by Bohnson and Robertson (5) in their study of the catalytic decomposition of hydrogen peroxide by iron and copper salts. This uniformity of the coefficients, however, disappears in experiments where a greater range of peroxide concentrations has been tested, especially at temperatures below 10°C. (see Table III).

The tendency of the coefficients to greater variation at extreme H_2O_2 concentrations and especially with increasing temperature is shown by Table III. The least variability in this series as in the previous was obtained with a 0.72 N concentration, the average value in this case being 4.33. This is shown even more strikingly when the coefficients are calculated over different temperature ranges, as is done in Table IV.

From Issajew's data (3) the temperature coefficient for yeast catalase can be calculated for a range of temperature of 0–10°C.

TABLE II.

Temperature range.	H_2O_2 concentration.		
	0.37 N	0.56 N	0.74 N
°C.			
10–20	3.19	4.76	3.98
10–30	3.69	4.03	3.98
20–30	4.32	4.28	3.82

TABLE III.

Temperature range.	H_2O_2 concentration.			
	0.36 N	0.72 N	1.44 N	1.80 N
°C.				
3.0–24.5	2.69	4.45	4.67	5.36
11.5–24.5	3.53	4.28		5.75
19.8–24.5	5.89	4.36	5.01	6.46

TABLE IV.

Temperature range.	H_2O_2 concentration.			
	0.36 N	0.72 N	1.44 N	1.80 N
°C.				
3–11 5	1.76	4.73	4.28	
3–15.8	2.08	4.39	4.85	4.31
3–19.8	2.15	4.51	4.64	5.05
3–24.5	2.69	4.45	4.72	5.62

TABLE V.

Temperature range.	H_2O_2 concentration.		
	0.3 N	0.4 N	0.5 N
°C.			
1–10	0	2,100	4,200
10–20	2,400	9,100	13,200
20–30	19,500	25,000	25,000

and is found to vary from 1.50 to 1.42, depending upon the peroxide concentration. His findings, thus, correspond more or

less to the value we obtained for a temperature range of 3–11.5°C. with 0.36 N hydrogen peroxide, which furthermore corresponds to the value (1.5) given by Senter (6) who experimented exclusively with small peroxide concentrations. It is clear, however, that the values recorded by either Issajew or Senter cannot represent anything but a very special condition and have no general significance. Except when a definite amount of hydrogen peroxide reacts with a definite quantity of catalase, the values of the van't Hoff coefficient vary considerably with increasing temperature. We regard, therefore, our value of about 4 as representing more nearly the average temperature coefficient.

Arrhenius' factor for the velocity of reaction increment with temperature, μ, has also been worked out for several series of experiments. The constants for the catalase reaction have been determined by Northrop's procedure (7). The values for μ obtained for different experiments, while showing considerable variation among themselves, nevertheless display great uniformity in the general trend of the change. A single example (Table V) will suffice to demonstrate this point.

It is obvious, thus, that the value for μ recorded by Senter, namely 6200, is really without significance since μ increases with a rise in temperature besides being affected also by the concentration of the hydrogen peroxide. The conclusion to be drawn is that the catalase reaction proceeds at an accelerated velocity as the temperature rises.

SUMMARY.

1. Increasing experimental temperature causes reduction in catalytic activity which is greater the higher the concentration of hydrogen peroxide. This reduction is the result of the destruction of catalase by the hydrogen peroxide, hence the maximum activity occurs at low temperatures where the destruction is less.

2. The catalase activity is greatest at temperatures between 0 and 10°C. The loss of activity between 10 and 20°C. is much smaller than between 20 and 30°C., while between 30 and 40°C. the loss is so rapid that the catalase reaction is very quickly exhausted.

3. Lowering the experimental temperature and increasing the

hydrogen peroxide concentration flattens out the curve of the catalase reaction until it becomes, at very low temperatures, a straight line. The slope of the curve varies inversely with the hydrogen peroxide concentration.

4. Under the combined action of low temperature and high peroxide concentration there appears a quiescent period which, following an immediate reaction lasting a minute or two, extends over a longer or shorter time depending upon the hydrogen peroxide concentration. Under these experimental conditions there is no appreciable latent period of reaction.

5. The van't Hoff temperature coefficient for the catalase reaction varies with the experimental temperature and hydrogen peroxide concentration. In our experiments with 0.72 N hydrogen peroxide the coefficient seems quite constant over a considerable range of temperatures and has a value of 4.

6. The Arrhenius' factor, μ, also increases with increasing temperature and with increasing hydrogen peroxide concentration. An increase in temperature produces an accelerated change in the catalase reaction velocity.

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STUDIES ON THE EFFECT OF TEMPERATURE ON THE CATALASE REACTION.

II. LOSS OF CATALASE ACTIVITY.

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Inactivation of Catalase by Heat.

In the preceding paper (1) the possibility of spontaneous inactivation of the enzyme by heat has been mentioned as a factor responsible for the diminished catalase activity, especially at temperatures above 20°C. To determine to what extent this may have played a rôle in the experiments at different temperatures, the activity of catalase subjected for various lengths of time to the influence of different temperatures has been investigated under standard conditions. Definite amounts of the catalase extract, measured into a series of crucibles, were quickly heated or cooled to the desired temperature and kept at that temperature for a certain period of time, usually an hour; the material was then quickly brought to room temperature and the amount of catalase present was determined by the oxygen set free from a known quantity of hydrogen peroxide. This was compared with the oxygen set free by the same amount of untreated catalase extract, the determinations being carried out under similar conditions (*cf.* Paper I) as far as pH and concentrations are concerned.

In the first experiments we studied the effect of temperature on the catalase at 10° intervals and the results, representing the final volumes of oxygen liberated in two series of experiments, are recorded in Table I.

Obviously catalase exposed for 1 hour to temperatures below 40°C. undergoes little or no inactivation, whereas at higher temperatures there is definite inactivation which increases with

an increase in temperature. The inactivation at 60°C. is practically complete, the greatest loss of activity occurring by exposure at temperatures between 40 and 60°C. We studied the

TABLE I.

Series.	Experimental temperature.	Catalase previously heated for 1 hr. to:					
		20°	30°	40°	50°	60°	70°
	°C.						
I	8.8	35.7	34.8	35.1	32.9	0	0
	20.0	35.5	34.0	34.4	27.3	0	0
	29.6	25.5	24.9	24.6	21.3	2.6	0
II	9.2	56.2	54.4	48.6	28.5	0	0
	21.0	39.0	40.0	37.4	20.4	0	0
	31.1	22.3	20.6	21.2	16.8	2.6	0

TABLE II.

Experimental temperature.	Catalase previously heated for 1 hr. to:					
	20°	40°	45°	50°	55°	60°
°C.						
8.9	50.7	50.0	48.4	47.0	34.5	0
20.2	41.2	37.5	35.2	31.5	27.0	0
29.5	23.3	21.7	22.3	18.6	12.0	0

TABLE III.

Catalase Activity Determined at 20°C.

Catalase heated for 1 hr. to:						
20°	50°	52°	54°	56°	58°	60°
37.1	28.6	26.1	23.8	?	0.9	0

TABLE IV.

Catalase Activity Determined at 19°C.

Catalase previously exposed for 1 hr. to:					
20°	5°	0°	− 5°	− 10°	− 15°
41.8	41.8	41.6	41.0	40.2	37.1

temperature at which the maximum inactivation takes place within 1 hour by limiting the intervals to only 5° with the result shown in Table II.

We can see from this experiment that complete inactivation must take place by an hour's exposure at temperatures between 55 and 60°C., since at 55°C. in the same length of time the inactivation is only partial. With the facilities at our disposal we could not determine the exact temperature causing the complete loss of activity in 1 hour but we performed a series of experiments wherein the effect of heating to temperatures 2° apart was studied. These experiments (Table III) show that maximum inactivation is really associated with a very narrow range of temperature change.

It is well to point out in this connection that we also studied the effect of low temperatures on the activity of the catalase, and found little or practically no alteration in this activity until the enzyme had been exposed for 1 hour to a temperature 15° below zero. This experiment is important as it demonstrates that the reduced activity observed in experiments carried out at 0° as compared with the activity of the same catalase concentration tested at temperatures between 0 and 10°C. cannot possibly be due to a spontaneous inactivation of the catalase (*cf.* Paper I). Even at −15°C. the observed loss of activity is not greater than that caused by exposure for 1 hour at 50°C. as can be seen from the experimental results in Table IV.

We next determined the rate of inactivation of catalase at 50, 55, and 60°C. We have shown already that exposure for an hour at the latter temperature is sufficient to cause complete inactivation, while at 55° for a similar period the inactivation is about two-thirds complete. Since, however, a certain amount of inactivation must already occur during the brief interval while the temperature is being brought up to the desired level we used in these studies of the rate of inactivation a different standard from that employed in the previous tests. The degree of inactivation, instead of being compared to that of catalase kept at 20°, was measured by referring to the activity of a similar sample which had been warmed to the same temperature as the other samples and then immediately cooled to room temperature. This procedure is safer not only because it corrects for the inactivation incident to the preliminary alteration in temperature until the desired level is attained, but it also permits a more exact timing of the entire process. In the last paper of this

series, when we discuss the latent period of the catalase reaction, we shall come back to this matter of the inactivation process. Here we shall merely record the results of these experiments (Table V).

It is clear from these experiments that at 50°C. the inactivation of the catalase is only partial even after heating for 2 hours and 20 minutes and does not apparently extend beyond about 25 per cent of the initial strength of the preparation. At 55°C. about 60 per cent of the catalase is inactivated in 1½ hours, but since the same degree of inactivation is attained within 1 hour it seems that this is practically the maximum inactivation at

TABLE V.

Catalase heated at 50°C. for:

0 min.	20 min.	40 min.	60 min.	80 min.	100 min.	140 min.
39.6	38.1	33.6	34.0	30.3		
40.7	35.8		33.2		34.7	30.6

Catalase heated at 55°C. for:

0 min.	5 min.	15 min.	30 min.	35 min.	45 min.	60 min.	65 min.	90 min.
38.3	36.0			23.6			21.9	15.8
35.9		26.7	18.7		18.7	16.0		

Catalase heated at 60°C. for:

0 min.	5 min.	15 min.	30 min.	45 min.
31.8	13.8	5.2	2.6	0

this temperature. At 60°C., on the contrary, the inactivation progresses very rapidly, being very nearly completed in 30 minutes, and entirely completed after 45 minutes of heating.

Fig. 1 represents a series of curves of the reaction obtained with unheated catalase and with catalase heated to 60°C. for 5, 15, and 30 minutes. This shows very striking changes not only in the total catalase activity but also in the character of the curves under these different conditions. An analysis of the curves in Fig. 2 shows that the inactivation of the catalase at 55 and 60°C. follows a definite course. We have attempted to fit these curves to various types of conical sections without much success; however, the experimental curves seem to correspond

closely to a theoretical curve calculated from the experimental data on the assumption that they represent points of a bimolecular isotherm.

We do not know what particular significance this may have, but it seemed worth calling attention to this fact, since the bimolecular formula gives the only mathematical expression which fits the experimental data, as can be seen from Fig. 2 where

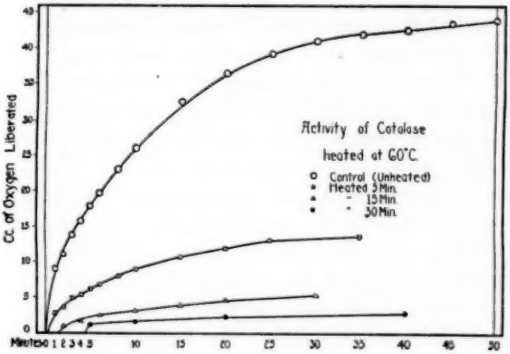
Cc. of Oxygen Liberated
Activity of Catalase
heated at 60°C.
Central (Unheated)
Heated 3 Min.
" 15 Min.
" 30 Min.
Minutes 1 2 3 4 5 10 15 20 25 30 35 40 45 50

FIG. 1.

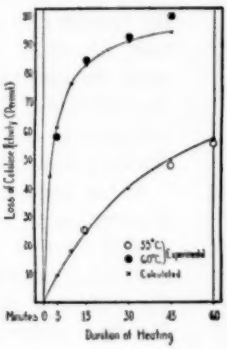

FIG. 2.

FIG. 1. Curves showing the course of the reaction with unheated catalase and with catalase samples heated for various lengths of time to 60°C. The concentrations and other experimental conditions are the same in all four experiments.

FIG. 2. Curves showing the inactivation of catalase heated for various lengths of time at 55 and 60°C. The ordinates give the percent of activity lost, and the abscissæ the duration of heating in minutes.

the experimental points are placed in relation to the theoretical curve drawn from calculated values. Northrop (2) found that the spontaneous inactivation of trypsin follows the monomolecular isotherm if the enzyme is highly purified. With less pure preparations the course of inactivation is bimolecular. Since our preparation of catalase is only partially purified, this may perhaps account for the circumstance that the heat inactivation follows a bimolecular course.

Influence of Temperature on the Destruction of Catalase by Hydrogen Peroxide.

We have learned from the previous paper that catalase is destroyed by hydrogen peroxide, the destruction increasing with temperature. It is not possible to measure directly the catalase destruction in any one single experiment, but this can be estimated from a series of experiments. The differential temperature effect furnishes us the means of measuring, at least indirectly, the rate of the catalase destruction. The rate of oxygen evolution, by which the catalase reaction is measured, really represents the resultant of two distinct phenomena: (1) the catalytic decomposition of hydrogen peroxide, and (2) the destruction of catalase by the hydrogen peroxide. The equilibrium between the two processes is determined by temperature.

It has been shown by Morgulis (3) and by Northrop (4) that the oxygen set free is a measure of the active catalase. A study of the reaction at different temperatures gives us, therefore, a method of attacking the problem of the catalase destruction in the course of the reaction. In a series of experiments, where the only variable is the temperature factor, the final amount of oxygen liberated is a measure of the relative concentration of active catalase under these conditions. It has been shown that the higher the temperature the smaller the active concentration owing to the more rapid destruction by the peroxide of the catalase initially present.

Thus, in one set of experiments we were able to calculate that, compared to the activity of the catalase at 10°C., there has been destroyed in the very first few minutes of the reaction 20 per cent at 16°C., 36 per cent at 20°C., 39 per cent at 25°C., and 41.6 per cent at 30°C. The total loss of catalase activity by the conclusion of the reaction at these four different temperatures was 25, 39.5, 46.3, and 55.6 per cent respectively. The very great destruction of the catalase at the very beginning of the reaction is, therefore, evident. This, of course, is not surprising since the hydrogen peroxide also disappears during the reaction, the greatest concentration being present during the early stages.

As the destruction at temperatures somewhat below 10°C. is

negligible or practically nil, we may use this active catalase concentration as a standard for comparison and thus obtain the relative catalase activity at various other temperatures. We can illustrate this with a few specific examples, taking the maximum results obtained for each separate series (at about 10°C.) as representing a unit of catalase activity. The catalase activities under various conditions are represented in Table VI. Although each of the series reported in Table VI is an independent set of experiments, there is remarkable agreement in the results, be-

TABLE VI.

H_2O_2 concentration.	Relative catalase activity.		Catalase destroyed.	
	20°C.	27°C.	20°C.	27°C.
N			*per cent*	*per cent*
0.2	0.98	0.83	2	17
0 3	0.89	0.57	11	43
0.4	0.72	0.43	28	57
0.5	0.68	0.42	32	58
	20°C.	30°C.	20°C.	30°C.
0 3		0.68		32
0 4	0.75	0.46	25	54
0.5	0.66	0.38	34	62
	19.4°C.	30°C.	19.4°C.	30°C.
0 36	1.00	0.47		53
0 54	0.74	0.48	26	52
0 72	0.67	0.45	33	55
0 90	0.58	0.43	42	57
1 08	0.50	0.39	50	61

traying a definite regularity in the extent of the catalase destruction in relation both to the experimental temperature and to the peroxide concentration.

But these results tell us as yet nothing of the course of the catalase destruction. We may, however, get light upon this process by extending the principle of the proportionality of the catalase to the oxygen liberated (the principle which has been defined by Northrop (4) mathematically) to other stages of the reaction as well as to the terminal stage. Since the velocity of the reaction at different temperatures varies greatly, such a

direct comparison of corresponding stages meets with considerable difficulty. This, however, can be overcome by superimposing the curves of the reaction at different temperatures in such a manner that the beginning and end of each curve exactly coincide. This can be done by plotting the curves with different time units on the abscissa, these units being proportional to the respective reaction velocities. Referring now to the curve of the experiment at the low temperature as the standard, the relative catalase activity at different times can be calculated from the heights of the ordinates at corresponding points, and thus the degree of destruction at those times determined for

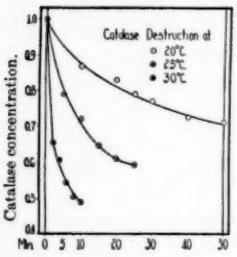

FIG. 3.

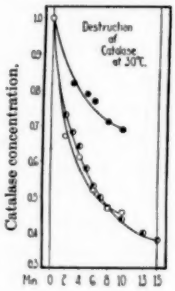

FIG. 4.

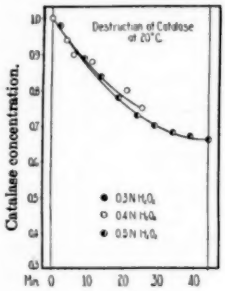

FIG. 5.

FIG. 3. Curves showing the rate of catalase destruction at 20, 25, and 30°C. The ordinates represent catalase concentration.

FIGS. 4 and 5. Curves showing the rate of inactivation of catalase at 20 and 30°C., respectively, and with 0.3, 0.4, and 0.5 N hydrogen peroxide.

the different temperatures. Although such indirect procedures are generally fraught with obvious dangers, the strikingly similar results obtained by this method under a great variety of experimental conditions lends considerable assurance to this as a valuable aid for the study of this problem.

The data presented graphically in Figs. 3, 4, and 5, show that the catalase destruction during the reaction follows a very definite course, the rate varying with temperature. In one series of experiments the velocity of the catalase destruction was two to three times as great at 25°C. as at 20°C., while at 30°C. it was eight to ten times as great. The data fit the monomolecular

TABLE VII.

0.3 N H_2O_2, 27°C.			0.4 N H_2O_2, 27°C.		
t	K	$A-x$	t	K	$A-x$
2	0.0355	0.85	5	0.0084	0.91
2.5	0.0330	0.83	10	0.0058	0.88
3.5	0.0300	0.80	13	0.0065	0.83
5	0.0317	0.70	17	0.0050	0.80
6.5	0.0315	0.63	20	0.0052	0.79
8.5	0.0296	0.56			
10	0.0301	0.45			
0.4 N H_2O_2, 20°C.			0.5 N H_2O_2, 20°C.		
4	0.0064	0.94	2	0.0060	0.98
8.5	0.0054	0.90	9	0.0055	0.89
12.8	0.0045	0.88	14	0.0056	0.84
17	0.0040	0.86	19	0.0057	0.78
21.3	0.0046	0.80	24	0.0056	0.73
25	0.0048	0.75	29	0.0054	0.70
			34	0.0050	0.68
			39	0.0045	0.67
0.5 N H_2O_2, 20°C.			0.5 N H_2O_2, 27°C.		
5	0.0087	0.91	5	0.0078	0.92
8	0.0156	0.75	13	0.0058	0.84
10	0.0140	0.73	20	0.0059	0.78
12	0.0110	0.71	26	0.0050	0.74
15	0.0109	0.69			
18	0.0106	0.66			

TABLE VIII.

Value of μ for catalase destruction.

Series.	Temperature range.	H_2O_2 concentration.		
		0.3 N	0.4 N	0.5 N
	°C.			
I	20–27	45,000	41,600	57,800
II	20–30		33,700	40,400
		0.37 N		0.55 N
III	20–25	40,500		28,400
	25–30	41,600		42,400
	20–30	41,000		35,000

equation (in a number of instances with remarkable precision) so that the values calculated from the average velocity constant agree very well with the values obtained by the method described above. A few examples will suffice to demonstrate the magnitude of the values of K from the familiar equation $K = \frac{1}{t} \log \frac{A}{A - x}$, where $A = 1$ represents the initial concentration of active catalase and $A - x$ represents the catalase concentration at various times t during the reaction (Table VII).

The rate of destruction of catalase by the hydrogen peroxide depends upon temperature. The Arrhenius factor, μ, for the effect of temperature has been calculated for several series of experiments with the results shown in Table VIII.

A comparison of these μ values with those recorded in Table V, Paper I, for the catalase reaction indicates a much greater uniformity in the case of the catalase destruction at different temperatures; it is also clear that in the latter instance these values are of an entirely different magnitude, even the smallest value being much greater than the largest μ value observed for the catalytic decomposition of hydrogen peroxide by catalase.

SUMMARY.

1. Exposure of catalase to temperatures up to 40°C. for 1 hour causes practically no inactivation. Inactivation begins at about that temperature reaching a maximum at about 58°C.

2. The rate of inactivation at 55 and at 60°C. seems to follow the course of a bimolecular reaction.

3. The inactivation at 50°C. even over a long period does not exceed 25 per cent of the catalase, at 55°C. 60 per cent, whereas at 60°C. the inactivation is complete in less than 45 minutes.

4. Cooling the catalase does not reduce its activity until −10°C. is reached. But even at −15°C. an hour's exposure occasions only about 12 per cent loss of activity.

5. Hydrogen peroxide destroys catalase, the rate of destruction increasing with an increase in experimental temperature and in hydrogen peroxide concentration.

6. The destruction of catalase follows the course of a monomolecular reaction.

7. The value of the Arrhenius factor μ is very large and quite constant as compared to those values obtained for the catalytic decomposition of hydrogen peroxide by catalase.

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STUDIES ON THE EFFECT OF TEMPERATURE ON THE CATALASE REACTION.

III. TEMPERATURE EFFECT AT DIFFERENT HYDROGEN ION CONCENTRATIONS.

IV. A THEORY OF THE CATALASE REACTION.

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Effect of Temperature at Different pH on Catalase Activity.

It has been shown by a number of investigators (Michaelis and Pechstein (1), Sörensen (2), Bodansky (3), Morgulis (4), Biéchy (5)) that the catalase reaction is greatly depressed in an acid medium. The study of the influence of changes in hydrogen ion concentration on the catalase reaction over a wide range of pH is beset with practical difficulties owing to the fact that a variety of buffers is needed for this purpose, and this brings into play the influence of different salt content which affects the final quantitative results. It frequently becomes impossible to tell whether the observed quantitative effect is due primarily to the change in pH or to the change in salt composition of the mixture. The direct adjustment of the reaction of the mixture by means of acids and alkalies is, in our opinion, a still less desirable procedure, and is not to be preferred to the use of appropriate buffers since it does not remedy the situation arising from a difference in salt content and composition, and is generally less reliable. We employed the Kolthoff series of buffers (6) covering a range from pH 3 to pH 9, and for the experiments where greater alkalinity was tested we used Sörensen's glycine-NaOH buffer which covers a range from pH 9 to practically pH 13. However, we also experimented with mixtures directly adjusted to various pH from 8 to about 14 by means of NaOH. In general, we can

corroborate the earlier findings, namely that a strong catalase reaction occurs over a range of pH 6 to pH 8; and, considering that the catalase activity is very little inhibited even at pH 5 or pH 9, it may be said that there is really no optimum pH for catalase activity in the strict sense of the word. Catalase acts well over an extent of hydrogen ion concentrations ranging from pH 5 to pH 9, but its activity diminishes decidedly on either side of these limits, the diminution being much more abrupt on the acid than on the alkaline side. Thus, at pH 4 one-half of the activity is still present, at pH 3 only one-fifth to one-seventh, and at pH 2 the activity is completely lost. On the alkaline side the loss of activity does not proceed quite so rapidly and the diminution up to pH 13 is gradual and continuous, but even at pH 13 considerable activity still remains (at least one-half). At pH 14 the catalase activity is completely suppressed. Bodansky (3) found that the catalase activity was entirely inhibited at pH 13, but we were unable to corroborate this with our kidney catalase preparation even when we followed his method of adjusting the reaction by appropriate additions of NaOH to the reaction mixtures. Considering what has been said with regard to the inherent impossibility of securing perfectly uniform series of quantitative results over the entire pH range, we may, nevertheless, sum up our experience along this line by presenting a slightly schematized curve of catalase activity at different pH values and temperatures.

Rising very rapidly from about pH 2.5, the curve forms almost a plateau between pH 5 and pH 9 (more correctly this plateau has a small curvature) and slopes off gradually to pH 13, this being much more gradual at high than at low temperatures. Between pH 13 and 14 there is a very steep and abrupt drop in the curve since all activity ceases at pH 14. On the acid side, more particularly up to about pH 3.5, there is comparatively very little difference in activity under various temperatures.

By far the greatest temperature effect is manifested over a range of pH 5 to pH 9, *i.e.* over hydrogen ion concentrations associated with the greatest catalase activity, thus demonstrating that the largest catalase destruction occurs under conditions most conducive to catalase activity. This fact tallies also with the observation recorded in Paper I of this series (7) that the

destruction of the catalase increases parallel to the reaction velocity. We must conclude, therefore, that either the part of the catalase molecule which causes decomposition of the hydrogen peroxide is also readily subject to destruction, or that the destruction of catalase by oxidation is greatly enhanced by the presence of OH ions in the system.

Furthermore, a glance at the curves in Fig. 1 leaves no doubt that although the catalase activity diminishes on either side of

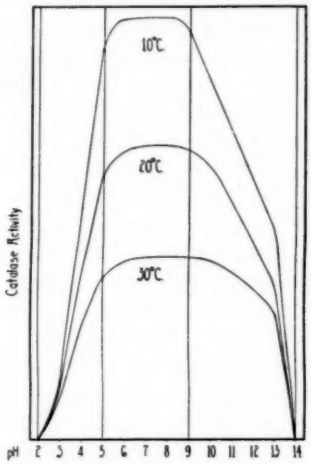


FIG. 1. This represents the catalase activity at 10, 20, and 30°C. over a range of pH values from 2 to 14. The curves are based upon many experiments and represent therefore composite curves slightly schematized in that they have been drawn to coincide as nearly as possible with the average for the experimentally determined points.

the pH range of 5 to 9, the effect at pH 2 to 5 is obviously radically different from that taking place at pH 9 to 14. The catalase at a pH less than 5 undergoes an alteration which manifests itself, for one thing, in a loss of sensitivity towards temperature effects, whereas on the alkaline side, even at pH 13, this effect is still very pronounced. Apart from this, however, the influence of an excess of either H or OH ions manifests itself in another, even more essential and fundamental manner, not revealed in Fig. 1,

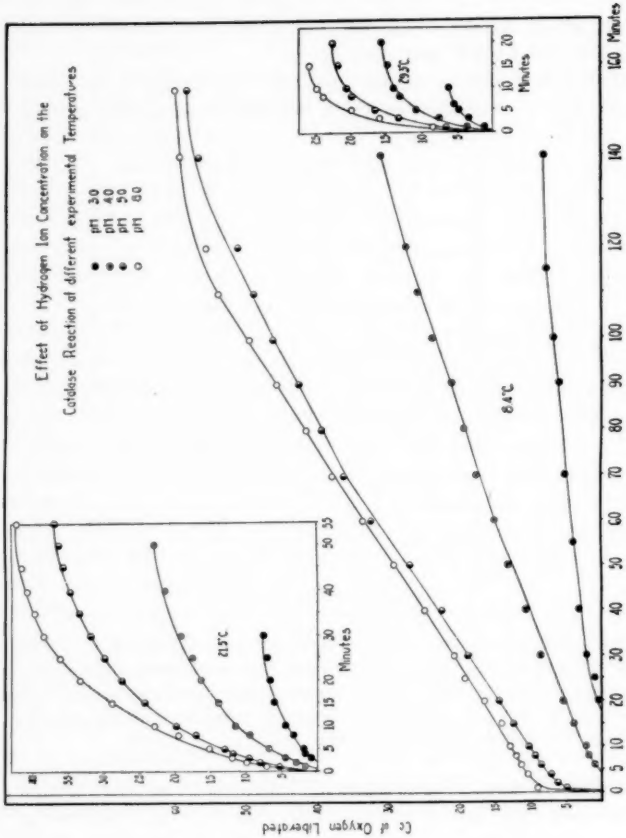


FIG. 2. A series of curves showing the course of the catalase reaction at different temperatures and pH of the mixture. The concentration of catalase and of hydrogen peroxide is the same in all these experiments.

but shown by the curves in Fig. 2; namely, a difference in the latent period of the reaction. This point, however, will be discussed fully in the next section.

Michaelis and Pechstein (1) suggested that catalase is an ampholyte which ionizes with the formation of both anions and cations. The molecule is assumed to be active, and the activity attains almost an optimum at the isoelectric point. On the acid side of this isoelectric point catalase fails to decompose hydrogen peroxide because it is present in the form of the catalytically inactive cations. The anion is assumed to be also active as the molecule, this assumption being necessitated by the fact that the catalase activity continues to increase even beyond the isoelectric point. This argument will hardly bear serious criticism. In the first place, the location of the isoelectric point of catalase is a purely gratuitous assumption. But even granting the main premises of the argument, the cations being inactive, it is not clear how the formation of anions would account for the increased activity as the sum of active particles would still remain the same as at the isoelectric point. Nevertheless, the Michaelis and Pechstein interpretation, though undoubtedly erroneous in detail, must be recognized as essentially correct in principle. We are led to conclude from our studies of the combined effect of temperature and pH variations that catalase is active probably only in the form of anions. Plotting the results of several experiments in such a way as to represent *relative* activity at different pH and temperatures (the maximum activity of the catalase observed at pH 7 or 8 at each experimental temperature being taken as 1) we note, in Fig. 3, first of all, that the curves thus obtained bear unmistakable resemblance to typical dissociation curves. This is only true for the acid range of pH. The pH at which the *relative* catalase activity becomes one-half varies according to the experimental temperature. On the average this occurs at pH 4, but at high temperatures (above 20°C.) this shifts definitely to the acid side. Since we cannot assign any isoelectric point to catalase, the marked repression of its activity with increasing hydrogen ion concentration suggests that we may possibly be dealing with a weak acid whose dissociation constant is 1×10^{-4}. Michaelis and Pechstein (1) give to catalase a dissociation constant of 2.88×10^{-5}, which

would correspond approximately to the one we get for 20°C., namely 6.6×10^{-5}. The first value 1×10^{-4} is based on the average found over a large range of temperature variations. Biéchy (5) states that for potato catalase he found that $K = 1.42 \times 10^{-7}$.

Since at pH less than 5 catalase is rapidly inactivated, losing its activity entirely at pH 2, we conclude contrary to Michaelis and Pechstein that the molecule must be inactive. The fact that catalase activity is maintained at a high level over an extensive range of pH 5 to pH 9 likewise fails to support the contention of these authors that the catalase molecule as such is the active substance. On the contrary, it seems more reasonable to believe

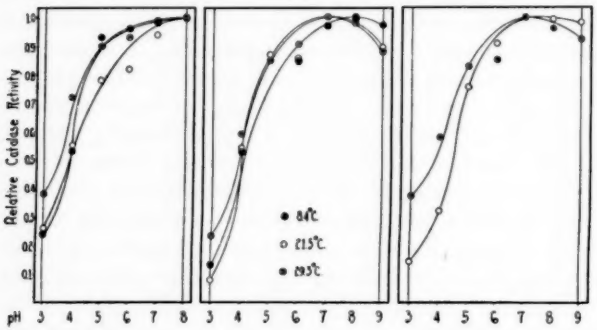


FIG. 3. A series of curves showing the *relative* catalase activity at different temperatures and pH. The maximum activity at pH 7 or 8 is taken as 1.

that if catalase ionizes the anion is the only active part. It also appears probable from our experimental results that the anion is very readily destroyed by the excess of hydrogen peroxide, the destruction increasing progressively with rising temperature. This is suggested by our observations already mentioned that the effect of varying temperatures gradually diminishes, ultimately to vanish almost completely, in the region below pH 4, where the concentration of anions must be very low and where a distinct inhibition of the catalase reaction takes place. It is likewise a striking fact, brought out by the curves in Fig. 3, that the temperature effect on the acid and alkaline side of the series is reversed. Thus, the *relative* catalase activity at pH 9 is

smaller at high than at low temperature, while at pH 3 the *relative* activity is greater at high than at low temperature. It is possible that this may be due to a shifting of the dissociation constant with a change in temperature, as was noted above. However, this fact bears out our contention further that the anion is the only catalytically active part of the catalase molecule, since the increased ionization, due to the higher temperature, evidently tends to offset the depression of ionization due to the large hydrogen ion concentration, and we should naturally expect this relative increase in catalase activity. On the alkaline side, however, due to the fact that the anion is readily attacked by peroxide and destroyed, the rise in temperature will tend to produce just the opposite effect, because owing to the greater concentration of the anion the rate of destruction of the catalytic agent will likewise increase. Furthermore, the high OH ion concentration may perhaps favor the destruction by oxidation.

From all our experimental results we are led to conclude that catalase may be a weakly acid substance, the catalytic activity of which is a function of its anions. Hence the rather abrupt and rapid onset of inactivation at pH less than 5. It is also clear that the destruction of the catalase, as is shown to occur by loss of its activity with rising temperature, is favored by increased alkalinity, and we are, therefore, led to conclude that very probably the anion is also the part most easily oxidized by the peroxide, this process increasing with the pH.

Latent Period of the Catalase Reaction.

During the first few minutes of the catalase reaction there are occurrences of far reaching importance which ordinarily escape attention unless the reaction is studied gasometrically whereby it may be observed continuously. We refer to the latent period of the reaction. Under ordinary experimental conditions (generally a pH of 7 and a temperature of about 20°C.) the latent period is so negligible that we have repeatedly overlooked it in our experiments, or were inclined to attribute the loss of a few seconds in the initiation of the reaction to purely extraneous influences. Under certain conditions, however, the latent period has attained such magnitude as to command our

attention very strongly, and this stimulated us to study this matter more closely. As was pointed out previously (4) under the combined influence of high hydrogen peroxide concentrations and low temperatures the catalase reaction undergoes an interesting modification: following an immediate spurt of catalase activity a quiescent period sets in, which is longer the higher the hydrogen peroxide concentration. This quiescent period is, of course, distinct from a latent period of reaction. There is no variation in the latent period in these experiments with varying peroxide concentrations at the same temperature. This fact is clearly brought out by the series of curves in Fig. 4 of Paper I, all curves issuing practically from the zero point.

In our experience, a distinct latent period becomes manifest when the catalase reaction is carried out under two particular experimental conditions. First, it becomes very prominent when catalase inactivated by heating for various lengths of time at 60°C. is studied. It was found then that not only did the activity diminish with an increase in the time of heating up to 45 minutes, at which time the enzyme was completely inactivated, but that a definite latent period appeared which became longer as the time of inactivation increased (see Fig. 1, Paper II (8)). Thus, after 5 minutes heating of the catalase sample 30 seconds were required before the reaction started; after 15 minutes heating, 90 seconds, and after 30 minutes heating the latent period increased to 270 seconds. It will also be noted from the Fig. 1 already referred to that there is a direct relationship between the degree of inactivation of the catalase material and the latent period of the catalase reaction.

The other experimental condition under which the latent period attains considerable importance is at pH less than 5, especially when the experiments are made at temperatures below 20°C. In fact, under such experimental conditions the latent period may become extremely prolonged (see Fig. 2). In Table I we record a number of observations on the latent period at different temperatures and hydrogen ion concentrations of the mixtures.

We do not record the latent periods observed at greater alkalinity since, on account of the use of different buffers, the results obtained at pH above 9 do not properly belong in the above series. However, we can say that over the entire range of

pH 9 to pH 13 we have failed to note any significant change from the magnitude of the latent period recorded in the table for pH 8 or 9. In fact, at pH 12 and 13 the latent period tends to become distinctly shorter than at lower pH values. The point we wish to emphasize in this connection is that in the case of high hydrogen ion concentrations the latent period also varies directly with the loss of activity of the catalase (at pH less than 5) and inversely with the reaction velocity. The increase in the magnitude of the latent period on the acid side with lowering of the experimental temperature is very striking, indeed.

Considering the great increase in the latent period of the catalase reaction under conditions whose principal effect is the inhibition of the activity of the catalase, we venture to suggest that there are two ways in which this enzyme may be altered, each manifesting itself in a reduction of the catalytic decomposition of hydrogen peroxide, but involving entirely distinct mechanisms. One type of alteration, we feel reasonably certain in assuming, is an actual oxidation of the catalase by the excess of peroxide, and we propose to designate this as the *destruction* process. This destruction increases with temperature as well as with the concentration of the hydrogen peroxide, and is also apparently dependent upon the OH ion concentration. This type of loss of activity has no effect upon the latent period of the catalase reaction. The other type of alteration manifests itself not alone in a reduction of catalase activity but also, and in a very striking manner, in a lengthening of the latent period of the reaction. We propose to differentiate this type of change as the *inactivation* phenomenon. It may be produced either through heating the enzyme, or it may result from an increase in hydrogen ion concentration beyond the pH of 5. The latent period becomes especially marked under the additional influence of low temperature. Assuming, as we obviously must, that at pH 4 or less the catalase is present principally as inactive molecules, we can readily understand why, especially when dissociation is reduced still further by lowering the temperature, the catalytic activity under such conditions is slow in getting under way. It is not improbable that a sufficient concentration of the active particles is required for the reaction to take place, and the more the dissociation of catalase is depressed by the combined

action of acidity (pH below 5) and lowering of the experimental temperature the longer will be the time necessary to reach that point. This interpretation is in exact agreement with the observed facts. We may likewise conclude that heat inactivation is a phenomenon involving some alteration of the catalase molecule which interferes with its dissociation. It is interesting to point out in this connection that material which was found completely inactivated by heating for 1 hour at 60°C., according to tests made at 20°C. or below that, would, in several instances, still produce a slight reaction when tested at 30°C. (*cf.* Table I, Paper II). Evidently, even after an exposure to heat for 1 hour

TABLE I.

Latent Period of the Reaction in Seconds.

pH	Experimental temperatures.					
	8.4°C.	9.8°C.	19.4°C.	21.5°C.	29.5°C.	31.1°C.
3	1120	560	165	130	26	12
				111	21	
4	240	216	30	32	18	6
				17	12	
5	18	20	35(?)	11	12	6
6	15	11	7	9	7	5
7	15	15	7	8	7	4
	8					
8	10					
	7					
9	10					

there is still enough dissociable material present in the catalase solution to produce a sufficient concentration of active particles under the proper temperature conditions to cause some catalytic decomposition of the hydrogen peroxide. We may, therefore, regard complete inactivation, either by heat or by acidity, as a condition under which the latent period of the catalase reaction has become infinitely large.

SUMMARY.

1. Catalase prepared from beef kidney shows strong activity at hydrogen ion concentrations ranging from pH 5 to pH 9, with the greatest activity being limited to the narrower range of pH 6 to pH 8.

2. At pH less than 5 the catalase activity is lost very rapidly and stops entirely at pH 2. The loss of activity between pH 9 and pH 13 is slow and much more gradual.

3. At pH 13 the catalase still has considerable activity (about one-half the original), but this is lost completely at pH 14.

4. The loss of catalytic activity with increasing temperature varies with the pH of the reaction mixture. The maximum destruction of catalase by rising temperature occurs within the range of pH of greatest catalytic activity.

5. At pH less than 4 the temperature effect almost entirely disappears, and the *relative* activity is greater at the higher temperatures. In mixtures with higher pH the *relative* activity diminishes with rising temperature.

6. Catalase seems to behave like a weak acid, the anion apparently being the catalytically active part.

7. A marked latent period appears when catalase has been inactivated by heating to 60°C. or when the reaction is studied at pH below 5 and at temperatures below 20°C. The latent period probably represents the time required for an accumulation of the active catalase dissociation product essential to start the decomposition of hydrogen peroxide, and is longer the less favorable the conditions for the dissociation.

IV. A THEORY OF THE CATALASE REACTION.

No theory of catalase activity can be built except upon the basis of an exact knowledge of the chemistry of hydrogen peroxide decomposition. Considering the paucity of this knowledge and in addition the complete ignorance of the chemical nature of catalase, the task is not materially different from that of solving an equation with two unknowns. The most that can be achieved in this state of the subject is to harmonize the known facts regarding the catalase reaction and to search for some modes of its behavior. With the principal elements remaining unknown, the representation of the reaction must necessarily be symbolic rather than definitive.

One can entertain little doubt that the first step in the enzymic decomposition of hydrogen peroxide must be a dissociation of the catalase into an active form. The evidence obtained from our study of the influence of varying pH favors the conception

that the active form may possibly be the anion of catalase, though one must not mistake this evidence for actual proof. Without stressing this point unduly we may, however, suppose that a change like this occurs: catalase→active catalase, which may be represented symbolically thus:

$$E_M \rightarrow E_A \qquad (1)$$

We may now consider the evidence for this assumption. At hydrogen ion concentrations exceeding 1×10^{-5} N this change is impeded with the result that the decomposition of hydrogen peroxide is diminished until it ceases altogether at a hydrogen ion concentration of 1×10^{-2} N. The change $E_M \rightarrow E_A$ is governed by temperature and pH, increasing with both. It is not unlikely that heating so alters the catalase that the change $E_M \rightarrow E_A$ is impeded with the result that 25 per cent of the catalase becomes unavailable after heating at 50°C., 60 per cent at 55°C., and at 60°C. the entire amount of catalase is thus affected. Since, however, the change $E_M \rightarrow E_A$ is promoted by a temperature rise we actually find that a catalase sample which, heated at 60°C., has apparently lost its activity completely upon being tested at temperatures of about 20°C. will still display a small activity when tested at 30°C. Similarly, when the change $E_M \rightarrow E_A$ is depressed by acidity, the catalase is *relatively* more effective at high than at low temperature. Finally, when the change $E_M \rightarrow E_A$ is impeded still more efficiently by a combination of factors, this manifests itself in a great prolongation of the latent period of the reaction. The occurrence of the latent period shows that a certain effective concentration of E_A is necessary to initiate the reaction with hydrogen peroxide. In this respect we can produce experimentally summation or antagonism of effects by proper combination of such factors as temperature and acidity. The quiescent period which, of course, is radically different from the latent period, is another manifestation of the same phenomenon. The quiescent period is the summation effect of low temperature and high hydrogen peroxide concentration. The slow transformation $E_M \rightarrow E_A$ in the cold makes the effective accumulation of E_A difficult, especially in the presence of an excess of hydrogen peroxide, and a quiescent period sets in, which varies with the hydrogen peroxide concentration, but this will be discussed later.

How does the active catalase E_A produce decomposition of hydrogen peroxide? What is the nature of this process? Actually the reaction is probably complex and proceeds in more than one step, but not knowing the intermediate stages, we may simplify this into the symbolic representation:

$$E_A + H_2O_2 \rightarrow E_R + \uparrow O_2 \qquad (2)$$

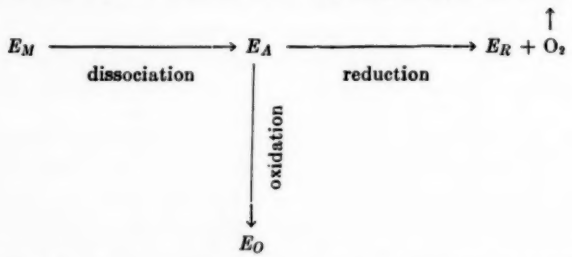
In other words, the setting free of oxygen from hydrogen peroxide involves (1) an oxidation of the H_2O_2, and (2) a reduction of the catalase to an inactive state. The latter change is apparently irreversible (Morgulis (9)). It is, of course, a familiar fact that hydrogen peroxide is a reducing as well as an oxidizing agent, and we attribute the liberation of oxygen by catalase to the oxidation of the hydrogen peroxide for several reasons. The prototype of reaction (2) is perhaps found in the reaction $Ag_2O + H_2O_2 = 2Ag + H_2O + \uparrow O_2$, where the hydrogen peroxide is likewise oxidized with the resulting liberation of oxygen and the Ag_2O reduced to metallic silver. It is noteworthy that when hydrogen peroxide functions as an oxidant no evolution of oxygen gas can be detected. Thus in our studies of the oxidation of uric acid by hydrogen peroxide (9) no formation of free oxygen has been observed unless catalase was added to the system. This is also easily demonstrated by a simple experiment. If to a neutral solution of KI is added a neutral solution of hydrogen peroxide there is an immediate liberation of iodine which colors the mixture yellow, but no oxygen is given off. In this step of the reaction the H_2O_2 is, of course, the oxidant, and 2 I^- is oxidized to I_2. It is only after the iodine has been liberated, following a brief but measurable interval of time, that oxygen is now set free, while the yellow coloration fades out owing to the disappearance of the iodine which oxidizes the hydrogen peroxide and is thus changed back to the iodide. The evolution of oxygen in this typical reaction of the catalytic decomposition of hydrogen peroxide is associated with a process of oxidation of the hydrogen peroxide (by iodine), but not with the oxidation of the iodide (by the hydrogen peroxide). We believe that the setting free of oxygen in the catalase reaction—the intimate

mechanism of which we do not as yet understand—is likewise the result of oxidation of hydrogen peroxide. There is no evidence but the conjecture is not perhaps too far fetched that a peroxide is formed as an intermediate compound, $E_A + 2H_2O_2 \rightarrow E_P + 2H_2O$, which is responsible for the oxidation of the hydrogen peroxide. But whatever the mechanism of this reaction may be, the oxidation of the hydrogen peroxide $E_A + H_2O_2 \rightarrow$ $\uparrow$ $E_R + O_2$, varies with temperature. Since it also depends upon the concentration of E_A it will be affected by hydrogen ion concentration in the sense that its course will be markedly repressed below pH 5.

Were there no complicating circumstances, the oxygen set free would be proportional to the concentration of E_M, but sufficient evidence has been found to show that E_A is being destroyed in the course of the reaction so that we are dealing with a changing concentration of E_A. This destruction is undoubtedly a process of oxidation

$$E_A + H_2O_2 \rightarrow E_O + H_2O \qquad (3)$$

and proceeds best at high temperatures, but increases also with a rise in hydrogen peroxide concentration and in OH ion concentration. We may now represent diagrammatically the changes in catalase incident to its reaction with hydrogen peroxide as follows:



Hence the obvious antagonism so far as the visible result of the reaction with the liberation of oxygen gas is concerned between the oxidation of hydrogen peroxide and the reduction of the hydrogen peroxide, the former alone resulting in oxygen evolution. The reaction $E_A \rightarrow E_O$ is slower than the reaction $E_A \rightarrow$

$E_R + O_2$ and frequently, especially at intermediate temperatures, does not become manifest until the reaction had already advanced so far that the concentration of E_A is very small as compared to the concentration of the still undecomposed hydrogen peroxide. In every reaction, therefore, there is a balance of effects which depends ultimately upon three factors—temperature, pH, and hydrogen peroxide concentration. It is possible by a suitable increase in the effective concentration of catalase (or diminished concentration of hydrogen peroxide) to produce even at high temperatures (35–40°C.) quick and complete decomposition of the hydrogen peroxide before any oxidation of catalase can occur. In other words, by proper adjustment of the relative enzyme and hydrogen peroxide concentrations and with the aid of temperature it is possible to make the reaction follow only one course:

$$E_M \rightarrow E_A \rightarrow E_R + O_2 \uparrow$$

On the other hand, by increasing the hydrogen peroxide concentration and the temperature it is possible to quicken the reaction in either direction. Since, however, the oxidation reaction has a greater critical thermal increment (*cf.* Table VIII, Paper II) the energy will be spent chiefly in destroying the catalase:

$$E_M \rightarrow E_A \rightarrow E_R + O_2 \uparrow$$
$$\downarrow$$
$$E_O$$

In this case the reaction $E_A \rightarrow E_O$ will be so quickened by the high temperature that only a small part of E_A will remain available to react with the hydrogen peroxide to produce oxygen. The oxygen produced under this condition is no longer affected by variations in hydrogen peroxide concentration. The same thing was shown in a less acute form in the experiments reported in Paper I (see Fig. 2). However, these striking results are only obtained at temperatures of 35°C. or above, and the reaction proceeds to completion with very great velocity (usually within 1 to 2 minutes). Owing to the distinctly explosive character of the reaction under such conditions it is not possible to measure its course.

We are now in a position to understand better the quiescent period, which we have seen to appear in the cold in the presence of a large hydrogen peroxide excess. Given a certain catalase concentration and a favorable pH (pH 7 in our experiments) the immediate reaction with the liberation of oxygen is determined by the concentration of free E_A. But following this initial spurt of activity during which the available E_A is all used up further dissociation $E_M \rightarrow E_A$ proceeds extremely slowly owing to the low experimental temperature, and the reaction at once slows down or, depending upon the relative concentration of the hydrogen peroxide in the system, stops altogether. We have shown already how the curve of catalase activity under these conditions tends more and more to approach a straight line (see Fig. 1, Paper I) the steepness of which varies inversely with the hydrogen peroxide concentration. Where the concentration of hydrogen peroxide is sufficiently great, the subsidiary reaction, $E_A + H_2O_2 \rightarrow E_O + H_2O$, tends to reduce the concentration of E_A and thus keep this below the effective level necessary for the initiation of the catalytic reaction, $E_A + H_2O_2 \rightarrow E_R + O_2$, and a quiescent period ensues which is longer the greater the concentration of the hydrogen peroxide. The quiescent period has therefore this in common with the latent period that both represent a lapse of time necessary for the active catalase E_A to attain an effective concentration. The differences between the two, however, are radical. Apart from the fact that the latent period precedes any activity, whereas the quiescent period follows an initial reaction, the former is associated with an impeded transformation $E_M \rightarrow E_A$, while the latter results from the subsidiary reaction $E_A \rightarrow E_O$, which constantly brings down the concentration of E_A in the system and thus prolongs the time necessary for the attainment of an effective concentration. By increasing sufficiently the hydrogen peroxide concentration, as was done in one experiment where this has been brought up to 18.8 N, it is possible to limit the catalase activity to just the immediate reaction of the available free E_A. In this case the reaction as far as the liberation of oxygen is concerned stops during the 1st minute, and the quiescent period which then ensues becomes infinitely long. This is,

therefore, similar to the condition previously suggested to occur under certain experimental circumstances (heating the catalase to 60°C. for over 30 minutes or lowering the pH to 2) when the change $E_M \rightarrow E_A$ is so completely hindered that the latent period also becomes infinitely long.

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SURFACE TENSION AS A FACTOR IN DETOXICATION.

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When foreign organic substances are introduced into the animal body they are rated as toxic or non-toxic according to the severity of the symptoms which they produce. The body protects itself against such harmful compounds by converting them as far as it can into less noxious substances or into products more easily eliminated (1). Toxicity considered as an effect may be ascribed to some definite chemical reaction, and the severity of this effect may be regarded as proportional to the product of the specific toxic nature of the compound and the amount of it present. It need not be a linear function of the concentration of the substance, but quantity certainly comes into consideration; hence factors which either mobilize the substance at points where it is harmful or remove it from such places are very important. In questions of the transference of these foreign substances in the animal body there must be considered the passage of the toxic compound through semipermeable membranes, hence factors increasing the concentration near the surfaces of such membranes are also significant; and at this point we encounter the question of the surface tensions of these substances in solution as a factor in detoxication. The possible significance of surface tension in this connection was first pointed out by Berczeller (2) according to whom detoxication of foreign compounds gives rise to products, the surface tensions of whose solutions are more like that of pure water, which are accordingly more easily excreted. This is quite true for the synthesis in the body of hippuric acid from benzoic acid and glycocoll, and it is also true for the two other detoxication reactions cited by him.

Detoxication reactions have for some time been of special interest in this laboratory, hence a large number of the products

of such reactions are available here in a pure form, representing all the types of detoxication. We felt it to be incumbent therefore on this laboratory to give this matter further study by measuring the surface tensions of some of the substances fed in our experiments as well as of the detoxication products to which they gave rise. We could then see if there was an agreement between these figures, interpreted according to Berczeller's theory, and our own recorded detoxication experience.

Although all the detoxication products so far encountered either reduce the surface tension of water or else have no appreciable effect thereon, their precursors cannot be classed by these quantitative effects on water as more toxic or less toxic materials, for some which are thought of as toxic are less effective in changing the surface tension of aqueous fluids than others classed as detoxication products and *vice versa*. Neither is there a constant relation in this respect when the substances fed and their detoxication products are listed in pairs. Those in which the surface tension change is in the direction of pure water are offset by an equal number of which the reverse is true.

In these measurements it was learned that for many compounds the decrease in surface tension varied considerably as to a time element, although it was quite constant for any given time interval; also that the solutions could be stratified and thereby give abnormal values. The selection of control conditions with respect to time interval and stratification is therefore very important. The readings on the instrument were made comparable by frequent stirrings of the solutions under observation and by making readings in the shortest possible time. Such agitated solutions in which the molecules have not yet definitely oriented themselves in surface layers are also thought to simulate more closely conditions in the flowing fluids of the body than those kept at rest until an equilibrium has been established.

The water used in all this work was laboratory distilled water redistilled first from alkaline permanganate and then from phosphoric acid, in order to free it from volatile organic compounds. Both redistillations were conducted in Pyrex glass. Surface tension measurements were made with the du Noüy tensiometer (3), adhering strictly to all the details of technique described by the author, excepting his "time drop." The scale readings of the

instrument were converted into dynes by the formula $\sigma = 0.6898\ R$, where R is the scale reading, and the coefficient is the constant determined by calibration of the instrument, which was done with weights certified by the Bureau of Standards. The observations were made at room temperatures ranging from 22–25°; this much variation in temperature was found to have no significant effect on the readings. The glass vessel containing the sample was cleaned with hot chromic acid mixture followed by washing and rinsing in the redistilled water. The solutions were prepared by adding the compound to 75 to 80 cc. of water at about 80° in a 100 cc. graduated flask. When apparently saturated this solution was set aside at room temperature for not less than 1 day and shaken vigorously from time to time to prevent supersaturation. Enough of the solution was filtered to give a 20 cc. sample. This was evaporated in a glass dish to determine the concentration of the solution. The evaporation was done on a water bath when the melting point exceeded 120°, otherwise in a current of filtered air. The solids were dried in a vacuum oven at 80° when the melting point exceeded 100°, otherwise over sulfuric acid at reduced pressure.

The sample to be measured for surface tension was agitated violently and decanted into the watch-glass with the minimum number of crystals consistent with expedience. A few crystals floating on the surface did not influence the readings. 2.5 to 3.5 cc. were used. The variation of volume between these limits had no appreciable effect on the readings. The fluid was stirred with the platinum ring between the observations, and the ring was flamed between tests. No less than three observations were made on each set, and several sets were run on each compound, some with days intervening. For each set of observations there was a set of surface tension readings made on the water used, and the average of two determinations was recorded. The values used in this study are the per cent decrease in the surface tension of water revealed by these pairs of determinations. Surface tensions were measured only on saturated solutions; nevertheless, with many of the more soluble substances the surface tension lowering can readily be seen to be due to the specific nature of the substances themselves, since many less soluble substances surpass in this respect some of the more soluble ones. Not much has

been done on the surface tension-concentration relation for non-electrolytes, hence extrapolation from the surface tension of saturated solutions to those of weaker solutions cannot be depended upon; but for our purposes here the concentration effect cannot be much of a factor on account of the obvious specific effect of the substances themselves. The following may be cited as conspicuous examples of this point.

Substance.	Concentration for 100 cc.		Decreases surface tension of water.
	mg.	*m.M*	*per cent*
Phenylpropionic acid	36	0.24	31
Phenylacetic acid	915	6.7	25
p-Bromophenylacetic acid	150	0.7	25
Benzoic acid	184	1.5	18
Phenaceturic acid	1145	5.0	13

The wide range of solubilities of the substances studied together with the difficulty of extrapolation to any given concentration caused us to use the solutions always at full saturation.

The fate of the following compounds in the body of the dog is the same as in the body of the rabbit (*cf.* Table I): benzoic acid, *o*-chlorobenzoic acid, *o*-nitrobenzoic acid, phenylacetic acid, *o*-nitrophenylacetic acid, phenylpropionic acid, cinnamic acid, picric acid, ornithuric acid, *p*-nitrophenylacetic acid, phenaceturic acid.

The fate of the following compounds in the body of the human being is the same as in the body of the rabbit (*cf.* Table I): benzoic acid, *m*-aminobenzoic acid, *p*-nitrophenylacetic acid, phenaceturic acid, phenylpropionic acid, cinnamic acid, ornithuric acid, *p*-hydroxyphenylacetic acid, picric acid (slight detoxication).

According to Berczeller, the surface tension of the conjugation product should be nearer to that of pure water than that of the original (toxic) substance fed; *i.e.*, the per cent *lowering* of the surface tension is greater for the substance fed than for the substance excreted. Tables I to III are so arranged that this relation is readily apparent. In Table IV is presented a summary of the results. The symbol + indicates that Berczeller's theory is upheld; the symbol − indicates that it is not upheld; the symbol 0 indicates that the substance was excreted unchanged. How this is to be interpreted will be discussed briefly in the conclusion of

TABLE I.

Detoxication by Rabbit.

Name of compound.	Concentration of saturated solution.	Surface tension of saturated solution.	Decrease in surface tension of water caused by saturation with the substance.
	mg. per 100 cc.	*dynes*	*per cent*
Substance fed, benzoic acid.	184	62	17
Excreted, hippuric acid.	463	71	3
Substance fed, *o*-chlorobenzoic acid.	6	69	16
Excreted, unchanged.		Same.	
Substance fed, *o*-bromobenzoic acid.	4.5	73	5
Excreted, unchanged.		Same.	
Substance fed, *m*-bromobenzoic acid.	49	69	9
Excreted, unchanged.		Same.	
Substance fed, *o*-nitrobenzoic acid.	772	59	16
Excreted, unchanged.		Same.	
Substance fed, *m*-nitrobenzoic acid.	717	66	8
Excreted, unchanged.		Same.	
Substance fed, *m*-aminobenzoic acid.	730	69	8
Excreted, *m*-acetylaminobenzoic acid.	100	69	13
Substance fed, phenylacetic acid.	915	58	25
Excreted, phenaceturic acid.	1145	65	13
Substance fed, *o*-chlorophenylacetic acid.	311	41	47
Excreted, unchanged.		Same.	
Substance fed, *p*-chlorophenylacetic acid.	222	55	20
Excreted, unchanged.		Same.	
Substance fed, *p*-bromophenylacetic acid.	150	59	25
Excreted, unchanged.		Same.	
Substance fed, *p*-hydroxyphenylacetic acid.	1732	57	27
Excreted, unchanged.		Same.	
Substance fed, *o*-nitrophenylacetic acid.	360	72	10
Excreted, unchanged.		Same.	
Substance fed, *p*-nitrophenylacetic acid.	180	68	16
Excreted, unchanged.		Same.	
Substance fed, phenylpropionic acid.	36	53	31
Excreted, hippuric acid.	463	71	3
Substance fed, cinnamic acid.	53	69	8
Excreted, hippuric acid.	463	71	3
Substance fed, picric acid.	1138	75	0
Excreted, picramic acid.	65	75	0
Substance fed, phenaceturic acid.	1145	65	13
Excreted, unchanged.		Same.	
Substance fed, ornithuric acid.	102	69	10
Excreted, unchanged.		Same.	

TABLE II.

Detoxication by Dog.

Name of compound.	Concentration of saturated solution.	Surface tension of saturated solution.	Decrease in surface tension of water caused by saturation with the substance.
	mg. per 100 cc.	*dynes*	*per cent*
Substance fed, *o*-chlorophenylacetic acid.	311	41	47
Excreted, *o*-chlorophenaceturic acid.	457	65	17
Substance fed, *p*-chlorophenylacetic acid.	222	55	20
Excreted, *p*-chlorophenaceturic acid.	117	67	12
Substance fed, *p*-bromophenylacetic acid.	150	59	25
Excreted, *p*-bromophenaceturic acid.	156	66	14
Substance fed, *m*-aminophenylacetic acid.	836	65	14
Exceted, unchanged.		Same.	

TABLE III.

Detoxication by Human Being.

Name of compound.	Concentration of saturated solution.	Surface tension of saturated solution.	Decrease in surface tension of water caused by saturation with the substance.
	mg. per 100 cc.	*dynes*	*per cent*
Substance fed, phenylacetic acid.	915	58	25
Excreted, phenylacetylglutamine urea.	839	45	40
Substance fed, *o*-chlorophenylacetic acid.	311	41	47
Excreted, *o*-chlorophenaceturic acid.	457	65	17
Substance fed, *p*-chlorophenylacetic acid.	222	55	20
Excreted, *p*-chlorophenaceturic acid.	117	67	12
Substance fed, *p*-bromophenylacetic acid.	150	59	25
Excreted, *p*-bromophenaceturic acid.	156	66	14

this article. Each entry in Table IV (excepting the cases noted) means that we have data on the surface tension both of the substance fed and of the detoxication product, if any.

TABLE IV.
Summary.

Substance.	Human.	Dog.	Rabbit.
Benzoic acid	+	+	+
o-Chlorobenzoic acid	a	0 (+)	0 (+)
o-Nitrobenzoic "	"	0 (−)	0 (−)
o-Bromobenzoic "	"	b	0 (+)
m-Nitrobenzoic "	"	a	0 (−)
m-Bromobenzoic "	"	b	0 (+)
m-Aminobenzoic "	−	"	−
Phenylacetic "	−	+	+
o-Chlorophenylacetic acid	+	+	0 (−)
o-Nitrophenylacetic "	0 (−)	0 (−)	0 (−)
m-Aminophenylacetic "	0 (−)	0 (−)	c
p-Chlorophenylacetic "	+	+	0 (−)
p-Bromophenylacetic "	+	+	0 (−)
p-Nitrophenylacetic "	0 (−)	0 (−)	0 (−)
p-Hydroxyphenylacetic acid	0 (−)	b	0 (−)
Phenaceturic acid	0 (−)	0 (−)	0 (−)
Ornithuric "	0 (−)	0 (−)	0 (−)
Phenylpropionic acid	+	+	+
Cinnamic acid	+	+	+
Picric "	−	−	−

a, not fed.
b, conjugates with glycine—no surface tension data on the conjugate.
c, acetylates—no surface tension data on acetylation product.

Totals.

\+ 17 cases.
− 6 "
0 26 " (4+, 22−).

CONCLUSIONS.

In order to interpret the foregoing data so as to apply them to Berczeller's theory we may group the cases under three categories: (*a*) clearly in its favor; (*b*) clearly against it; (*c*) not clearly for or against it. The last named category includes those instances where the ingested substances are excreted unchanged; and since this is so often the case, it is of importance to decide as well as we can how these instances are to be judged.

The significant paragraph of Berczeller's article as regards this point is as follows:

"Der tierische Organismus hat in seinen Zellen und auch in seinen anderen Struckturen eine ausserordentlich grosse Oberfläche; wenn eine Substanz die Oberflächenspannung erniedrigt, wird sie in diesen Oberflächen angehäuft; wenn sie diese Wirkung verliert, wird sie in der Oberfläche in kleinerer Konzentration vorkommen, und damit steht die Giftigkeit in engen Zusammenhange. Die obenerwähnten Substanzen sind so lange Gifte, bis sie in grösserer Menge in den Zell- und Struckturoberflächen vorhanden sein können, sie hören auf Gifte zu sein, wenn sie von dort verdrängt werden, einfach dadurch, dass sie eine solche chemische Veränderung durchmachen, durch die sie die oberflächenspannungserniedrigende Wirkung einbüssen."

As we interpret this statement, it stresses too much the quantitative and minimizes the qualitative (*i.e.* the specific) aspect of toxicity; and in saying that a substance that is toxic by reason of a rather high concentration on the cell surfaces becomes innocuous simply because it is changed into a substance with a higher surface tension, it seems to follow that all substances which are soluble enough to give a fair concentration and which reduce the surface tension of water absolutely speaking to a reasonable degree must "suffer such a chemical change as will cause them to lose their surface tension-reducing property." It does not seem unjustified to take this meaning from the article of Berczeller; and on this interpretation it is clear that the cases where fairly soluble substances whose solutions possess rather low surface tensions are excreted unchanged must be counted against his theory. That is, if a substance has a very high ratio of solubility to surface tension, it may be expected to be detoxicated by the body. The lowest value for this ratio for substances which actually are detoxicated in our observation is had for phenylpropionic acid and cinnamic acid. From this standpoint, substances whose ratio is conspicuously above this, yet which are excreted unchanged, do not behave in accordance with Berczeller's theory; and giving even the most liberal interpretation to these cases we have the twenty-six 0 cases divided into four which favor the theory and twenty-two which do not, giving a total of twenty-one cases for and twenty-eight against.

The many cases where the theory is substantiated show that

surface tension is often one of the important factors entering into detoxication; but our figures show that taken alone (as Berczeller seems to mean it to be taken) it is too mechanistic to be an adequate explanation either of the causes or of the processes of detoxication. Finally, as has been pointed out before (1), it does not explain why the same substance may behave altogether differently in the organisms of different animals.

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THE EFFECT OF DIHYDROXYACETONE ON INSULIN HYPOGLYCEMIA.

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The trioses have been thought to be products of the intermediary metabolism of the higher carbohydrates, and dihydroxyacetone has been alleged to be metabolized by the diabetic patient, successfully replacing as much as 40 units of insulin per day. Since the triose, dihydroxyacetone, can now be obtained in considerable quantity, the question of its use as a food for diabetics has been raised (1), both on account of its caloric value which is similar to that of other carbohydrates, and its antiketogenic action which permits a more liberal allowance of fats to be utilized. As the work of Allan (2) shows the action of insulin on carbohydrate to be a monomolecular reaction, resembling in this respect the action of adrenalin (3), it follows that the action of insulin occurs at one point in the metabolism of the carbohydrate, and other evidence goes to show that this point is located close to the glucose⇌glycogen stage. Lower derivatives of hexoses should be metabolized without the use of insulin, and proof that a substance can be so metabolized would furnish important evidence in favor of the view that it is in the path of metabolism of the carbohydrate in the body.

By a method recently devised (4) it can be shown that, under normal circumstances, dihydroxyacetone, if present in the blood stream at all, must be exceedingly minute in amount and, further, there can be little variation in the quantity throughout the day. The exceedingly rapid disappearance of administered dihydroxyacetone from the blood stream of normal individuals must indicate its conversion to some other substance since it is not excreted in the urine of these subjects. If synthesis to a more

complex molecule can be shown it would be against the view that this triose is an intermediate step in the metabolism of the carbohydrate.

Noble and Macleod (5), on normal animals, and Fletcher and Campbell (6), on diabetic patients, have shown that a number of substances, including glucose, mannose, fructose, cane sugar, maltose, lactose, galactose, starches, dextrins, and glycerol, are effective in controlling hypoglycemia due to overdosage of insulin. Other sugars, such as arabinose, xylose, rhamnose, and raffinose, as well as non-nutrient substances, are completely ineffective. The speed of recovery differs with the substance used and recovery does not ensue if the substance be administered in such a way that no hexose can be formed from it. By using substituted sugars, Herring, Irvine, and Macleod (7) have shown that recovery from insulin hypoglycemia only ensues when a hexose, or hexose derivative, containing (1) a reducing group in the first or second position (counting the primary alcohol group as six), (2) ring formation through the fourth carbon atom, (3) asymmetry in groups 3, 4, and 5, is present or capable of being produced in the organism. Cure of insulin hypoglycemia is thus limited to glucose, mannose, or maltose, or to substances capable of being synthesized into the above substances. Recovery of an animal from insulin hypoglycemia, therefore, furnishes *prima facie* evidence that the substance administered is converted to hexose (tentatively glucose) and thus indicates the path of its metabolism.

On numerous occasions dihydroxyacetone has been used to cure hypoglycemia in human diabetics in amounts varying from 10 to 50 gm., approximately the same doses as if glucose were the antidote selected. In general it may be stated that the smaller dose is less rapidly and less completely effective than glucose, particularly if the reaction be severe. In any case the restoration of the blood glucose is slower than with glucose itself though not so slow as with glycerol, with which this triose is closely allied. Its behaviour in relieving hypoglycemia closely simulates levulose which is also a ketose molecule.

When the smaller doses are given the dihydroxyacetone appears in the blood only in minute traces and in the urine not at all. With larger doses the amount found in the blood stream is

greater, actual increase in blood sugar occurs, and, occasionally, small amounts appear in the urine accompanied by glucose. When given to some diabetics taking insulin, but without reaction, the total blood sugar may be raised and a mixture of glucose and triose appears in the urine. Symptomatic recovery of the patient from insulin hypoglycemia is less rapid but just as effective when the triose is used as when glucose is used. The patient may later show traces of glucose in the urine as is seen after excessive treatment of the reaction with glucose itself.

In Tables I, II, and III are shown the progress of recovery from insulin hypoglycemia in diabetic patients. In one case the blood sugar increase is somewhat greater than usually occurs with the amount of dihydroxyacetone used. The amount of dihydroxyacetone found in the blood is, in many cases, larger than shown here. The results are apparently a function of at least three variables: the rate of absorption of the triose from the alimentary tract; the rate of its conversion into glucose; and the rate of storage or utilization of the glucose.

The clinical evidence of recovery from hypoglycemia rests on subjective and objective signs and symptoms and the chemical findings. In some patients astonishingly small changes in the actual blood sugar are associated with marked improvement in their clinical condition. Unlike our experience with non-nutrients (6), however, the patient who expresses subjective relief from hypoglycemic symptoms with triose continues to improve, whereas when non-nutrients are given the blood sugar continues to fall and the symptoms return.

With the clinical evidence in mind, it seemed desirable to study the condition further in relation to known single doses of insulin, administered in the same way, where the triose could be administered intravenously to eliminate uncertainties of absorption and where definite physical signs uninfluenced by subjective sensations might be taken as evidence of the severity of the hypoglycemia. Rabbits were used, as there exist many experimental data on the response of these animals to insulin and to glucose. A liberal overdose of insulin (10 units) was administered intravenously after securing an initial specimen of blood for blood sugar and dihydroxyacetone estimation. Blood sugar estimations were carried out by the Folin-Wu (8) method, using

Hypoglycemia in Diabetic Patients Cured by Dihydroxyacetone.

TABLE I.

Patient a severe diabetic recently in coma, receiving 60 units of insulin per day. Fasting blood sugar 0.145 per cent.

Time.	Blood sugar.	Blood dihydroxyacetone.	Remarks.
p.m.	*per cent*	*per cent*	
8.20			Severe reaction; semiconscious.
8.30	0.035	0	10 gm. dihydroxyacetone given.
8.50	0.064	0	Recovery commenced.
9.10	0.067	0	Completely cured.
9.30	0.072	0	No dihydroxyacetone in urine.

TABLE II.

Same patient as in Table I 5 days later.

Time.	Blood sugar.	Blood dihydroxyacetone.	Remarks.
p.m.	*per cent*	*per cent*	
3.45			Moderate reaction.
4.00	0.053	0	50 gm. dihydroxyacetone given.
4.20	0.091	0.004	Considerable improvement at 4.10 p.m.
4.40	0.161	0.004	Out of reaction.
5.00	0.215	0.004	No dihydroxyacetone in urine.

TABLE III.

Severe diabetic requiring 55 units of insulin.

Time.	Blood sugar.	Blood dihydroxyacetone.	Remarks.
p.m.	*per cent*	*per cent*	
8.30	0.029	0	Reaction; drowsy.
8.35			50 gm. dihydroxyacetone given.
8.55	0.042	0.003	Improved at 8.45 p.m.
9.15	0.056	0.013	Apparently normal.
9.35	0.066	0.009	Normal.
11.05	0.085	0.002	

1 cc. of blood; the dihydroxyacetone estimations by the method recently described by one of us (4). Urines were not examined in all instances. In many cases no evidence of hexose or triose

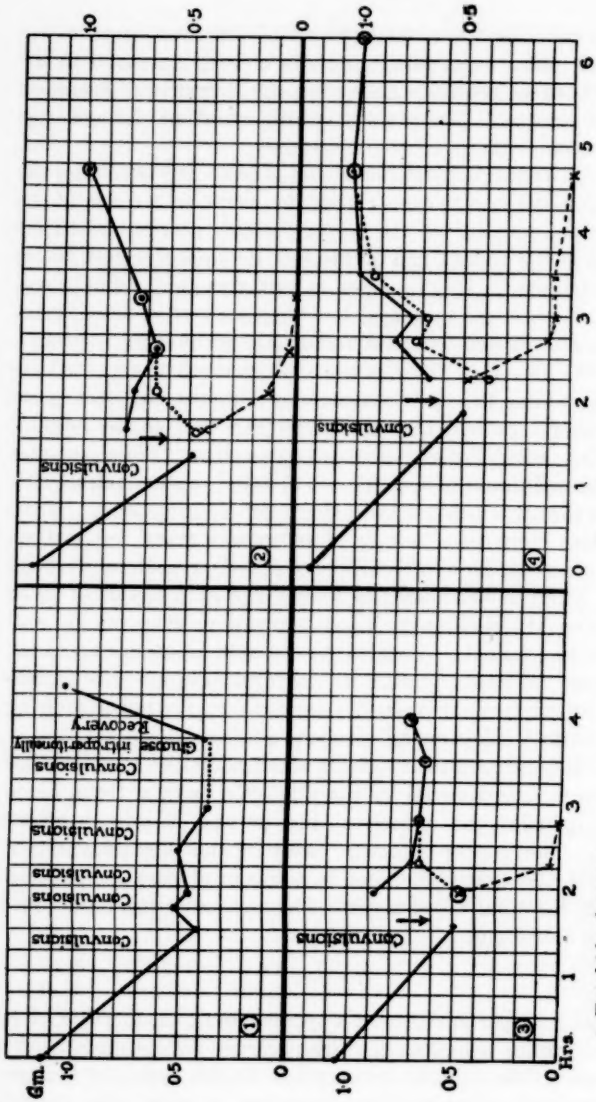
in the urine after the administration of dihydroxyacetone was found. In other cases small amounts of each appeared in the urine, the hexose probably only when the threshold of excretion level was reached. The point at which excretion of dihydroxyacetone occurs has not been determined.

As is well known to be the case with glucose, the results on the blood sugar fall into various classes according to the size of the insulin dose, severity of the hypoglycemia, previous nutritive condition of the animal, and duration of the hypoglycemia before cure is attempted. In some cases, as with glucose, the dihydroxyacetone administered produced but temporary relief from symptoms, the blood sugar rising but falling later as the excess of insulin used up the glucose. Since, in the experiments here reported the insulin and later the dihydroxyacetone administered were given intravenously through the one lateral ear vein of the animal, there is no possibility of failure of absorption. Blood samples were obtained from the opposite ear to avoid contamination with traces of the 10 per cent dihydroxyacetone solution used. When convulsions occurred a sample of blood was taken for analysis and 10 cc. of 10 per cent solution of dihydroxyacetone were injected. Further blood samples were taken at intervals of 15 to 30 minutes for the following 2 to 6 hours, depending on the condition of the animals. Control experiments, using insulin alone, and others in which glucose or dihydroxyacetone was administered subcutaneously were also carried out, the latter showing a well marked delay in the recovery process.

Since dihydroxyacetone reduces alkaline copper solutions, the blood sugar, as estimated by the Folin-Wu method (total blood sugar), includes the reduction due to any dihydroxyacetone present. The copper-reducing power of the dihydroxyacetone present is, therefore, calculated in terms of glucose and subtracted from the total blood sugar. The remainder has been called the true blood sugar.

Results.

Graph 1 (upper left) shows a typical result of injection of 10 units of insulin intravenously into a fasted 2 kilos rabbit. The blood sugar falls from its initial value, in this case 1.14 gm. per liter, to a level of 0.4 to 0.5 gm. per liter, at which time convul-



• Total blood sugar.
× Blood dihydroxyacetone.
○ True blood sugar.
→ Dihydroxyacetone given.

CHART I. Effect of dihydroxyacetone on insulin hypoglycemia in rabbits. Time in hours. Sugar content in gm. per liter.

sions occur. A slight increase in blood sugar occurs following a convulsion in some cases but rapidly returns to the convulsion level, and numerous convulsions succeed one another at short intervals to be terminated promptly by intravenous glucose administration.

The remaining graphs on Chart I show the results of administering 1 gm. of dihydroxyacetone intravenously to other animals similarly treated. This amount is usually sufficient, as shown in the graphs, to cause a complete cessation of the convulsions, though occasionally this dose is insufficient to give permanent relief as is also found in some instances when 1 gm. of glucose is injected. The animal in such instances recovers for a time; the blood sugar, however, slowly falls again and ultimately another convulsion occurs which may also be relieved or cured by the intravenous administration of 1 gm. of dihydroxyacetone or glucose.

Difficulty in obtaining suitable amounts of blood in rapid succession, together with the undesirability of excessive hemorrhage in these small animals, makes somewhat impractical a closer analysis of the immediate effect of introduction of the dihydroxyacetone. The effect, however, as in the human cases, differs in one particular from that of glucose. The exhilarating effect described by patients as "running through my veins like brandy," and shown by rabbits in coma or convulsions jumping from a table with full powers of equilibration before the intravenous injection of glucose is completed, is not duplicated by dihydroxyacetone. The effect is definitely slower and is never complete in less than 5 minutes, and often 10 minutes is required. Muscle weakness is first improved. The sprawling, disordered animal gathers its limbs close to the body; the pupils, if previously dilated, contract; the animal attempts to sit up and finally does so, maintaining balance with greater and greater efficiency; if made to move, walks like a cat with a poorly maintained and overcorrected equilibrium, then finally hops about normally. Voluntary attempts to regain the normal position are sometimes slow in appearing though the animal will at this time in response to external stimuli assume the sitting position or move about.

As shown in the chart, the total blood sugar rises after administration of dihydroxyacetone and tends to approach the

normal level in 2 to 6 hours. In such cases the animal recovers and presents no further symptoms of insulin overdosage.

The dihydroxyacetone content of the blood is greatest immediately following the injection, is still present in considerable amount 15 minutes afterwards, then rapidly falls, and 1 hour later is very small in amount if it has not entirely disappeared.

In spite of the blood sugar-reducing action of insulin the true blood sugar rises coincidentally with the fall in the dihydroxyacetone content of the blood, though naturally not to an equivalent extent, and finally constitutes the whole of the total blood sugar. Since the animal is without any other source than the dihydroxyacetone for the increasing glucose content of the blood, it may be safely inferred that the latter arises from the dihydroxyacetone administered, and constitutes further evidence that dihydroxyacetone is metabolized through glucose. There is no evidence to suggest that these results obtained on normal animals will not hold in diabetic animals as they do in humans, the subjects of diabetes mellitus. Further, if this reasoning be correct, the glucose so produced will require insulin in the further course of its metabolism. It seems probable, therefore, that under these circumstances dihydroxyacetone does not function as an intermediary product in the metabolism of glucose, and also, unless there exists a mechanism for utilizing glucose made from dihydroxyacetone more efficiently than other glucose when a limited quantity of insulin is available it seems difficult to accept the view that dihydroxyacetone can be metabolized in diabetes when other carbohydrates cannot be so metabolized.

The delay in the cure of insulin hypoglycemia by dihydroxyacetone over the time required for cure by glucose is undoubtedly to be ascribed to the necessity for the preliminary conversion of this substance to glucose, and recalls the similar behaviour of glycerol. The point at which this conversion is accomplished is a subject for future investigation.

SUMMARY.

Previously it has been abundantly shown that no substance inconvertible into glucose, mannose, or maltose can cure insulin hypoglycemia.

The triose, dihydroxyacetone, is shown to be able to cure insulin hypoglycemia both in man and animals, and is, therefore, a precursor of glucose.

Dihydroxyacetone also forms glucose in an animal, the subject of insulin hypoglycemia, in excess of the amount necessary to maintain the animal free from convulsions.

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SIMPLER NITROGENOUS CONSTITUENTS OF YEAST.

I. CHOLINE AND NICOTINIC ACID.*

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The problems presented by the metabolic changes which take place in the living cell are fundamentally chemical in nature. It has therefore seemed worth while to expend some time upon a study of the constituents of the cell in the hope that a fuller knowledge of the chemical environment in which the metabolic processes take place will eventually lead to a better understanding of what these processes may be. A recent study of the simpler constituents of the juice extracted from the alfalfa plant has impressed us with the inadequacy of our present knowledge of cell constituents and also with the difficulty of investigation in this field with our present methods. It seemed likely, however, that better results might be expected if a simpler organism were chosen for study.

Brewers' yeast was selected since it is readily available in relatively pure culture and in large quantities. Extracts from yeast have been studied in this laboratory in connection with the preparation of vitamin concentrates, and this work has emphasized the importance of investigation of the constituents of the yeast cell. In this paper a brief description is given of the method employed for the fractionation of the yeast extract, together with the results of the examination of the fraction which contained choline and nicotinic acid.

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The author wishes to express his appreciation of helpful advice from Dr. Thomas B. Osborne.

Top yeast, skimmed from the vats of a local brewery, was chilled with ice, stirred up with liberal amounts of ice-cold distilled water, and filtered with suction. The washing was repeated. As it required only a few hours and was carried out at low temperature, autolysis was kept at a minimum. The cakes of yeast were then disintegrated in a little water and the suspension poured into a large volume of boiling distilled water containing 0.01 per cent of acetic acid, thereby inactivating the enzymes almost instantaneously. The yeast was boiled for several minutes and filtered on folded papers. The extract was concentrated *in vacuo* to about one-fifth of its volume and treated with sufficient alcohol to make 53 per cent by weight. The voluminous precipitate, which consisted largely of inorganic matter, was discarded. The filtrate

TABLE I.

Yeast Extract Representing 6145 Gm. of Dry Yeast, Containing 464.8 Gm. of Nitrogen.

	gm.	*per cent of total N*
Total nitrogen	64.4	
Ammonia "	1.4	2.17
Amide "	2.8	4.34
Amino "	22.15	27.31
Organic solids	620.6	
Ash	263.6	
Phosphorus	53.8	

Organic solids are 10.38 per cent nitrogen.

was concentrated *in vacuo* and is referred to as "yeast extract" in the following pages. The extract prepared from yeast equivalent to 6145 gm. dried at 110°C., and containing 464.8 gm. of nitrogen, contained 14.39 per cent of the solids and 13.86 per cent of the nitrogen of the dry yeast. Extracts prepared in this way have been shown by Osborne and Wakeman (1) to be free from protein. The results, given in Table I, were obtained on analysis of the reddish brown solution.

The method of fractionation of this solution was substantially the same as that described in previous papers dealing with the constituents of alfalfa juice (2, 3). With the object of removing phosphoric and other acids forming insoluble barium salts, barium hydroxide was slowly added in cold saturated solution with

mechanical stirring until further addition yielded only a faint cloudiness. The reaction was distinctly alkaline to phenolphthalein at this point. The precipitate was removed and washed with cold saturated barium hydroxide solution.

Sufficient alcohol was added to the filtrate to make 33 per cent by weight. A small precipitate was produced which was centrifuged off. This behavior is in marked contrast to that of alfalfa filtrate. The barium hydroxide and alcohol precipitate from this material was very voluminous and contained considerable quantities of malic and malonic acid[1] together with nitrogenous substances. On the other hand the precipitate from yeast extract contained an insignificant proportion of the total solids, and it is therefore improbable that these or similar acids occur in yeast in any notable proportions.

The filtrate from the barium hydroxide precipitates was freed from reagents and treated with Neuberg's reagent (mercuric acetate, sodium carbonate, and alcohol) (4). The precipitate so obtained is now under investigation. The filtrate was acidified to Congo red with hydrochloric acid, mercury removed as sulfide, the solution concentrated *in vacuo*, and as much sodium chloride as possible removed by precipitation with absolute alcohol. The solution was concentrated and extracted with ether which removed a not inconsiderable amount of a crystalline substance that has not yet been investigated.

The solution was then treated in the usual way with phosphotungstic acid. The precipitate was decomposed with barium hydroxide and the solution found to contain 3.35 per cent of the total nitrogen of the yeast extract. An excess of hydrochloric acid was added and a considerable quantity of potassium, which had been precipitated by the phosphotungstic acid, was removed as chloride by means of alcohol.[2] The bases of the choline fraction were then precipitated by the addition of mercuric chloride in boiling saturated aqueous solution. The solution was chilled

[1] Unpublished data. In connection with the presence of malic and malonic acids in the alfalfa plant, see Turner, W. A., and Hartman, A. M., *J. Am. Chem. Soc.*, 1925, xlvii, 2044.

[2] This step is necessary. Otherwise the dangerously explosive potassium picrate will be obtained in the subsequent operations. See Osborne, T. B., Wakeman, A. J., and Leavenworth, C. S., *J. Biol. Chem.*, 1922, liii, 422.

overnight, the crystalline precipitate filtered off, the filtrate concentrated *in vacuo*, and treated as before with mercuric chloride. These operations were repeated and the final crop was taken from a volume of about 400 cc. The precipitates were decomposed with hydrogen sulfide and united. This solution, the choline fraction, contained 2.41 per cent of the nitrogen of the yeast extract.

Hydrochloric acid was removed from an aliquot part of this solution by means of silver sulfate, reagents were removed, and picric acid equivalent to the nitrogen added. Choline picrate and the picrate of nicotinic acid were readily obtained and separated by fractional crystallization from absolute alcohol and the latter substance purified by recrystallization from water. The choline accounted for 85.8 per cent of the total nitrogen of the fraction and nicotinic acid for 6.4 per cent. These figures are not precise, however, since the total nitrogen of the solution was determined by the Kjeldahl method and the nitrogen of nicotinic acid is only partially converted into ammonia by this method. Assuming that only one-third of the nicotinic acid nitrogen had been so converted in the determination of total nitrogen of the choline fraction, the yields of choline and nicotinic acid would be 82.5 and 6.2 per cent respectively. The error from this source is therefore small and we may safely assume that 89 per cent and probably somewhat more of the nitrogen of this fraction is accounted for by these two substances.

The filtrate from the mercuric chloride precipitate was freed from mercury and from hydrochloric acid in the usual way. Picric acid in small excess was then added and nicotinic acid equivalent to 13 per cent of the nitrogen of the fraction was obtained by fractional crystallization. Picric acid was removed from the mother liquors and the bases were again precipitated by means of phosphotungstic acid. Fractional crystallization of the picrates from this material gave a small additional quantity of nicotinic acid. Owing to the small quantity of material available no attempt was made to investigate the nature of the other substances in this fraction.

The yields of choline and of nicotinic acid obtained are given in Table II.

Both choline and nicotinic acid have been previously reported

as constituents of yeast. Meisenheimer (5) analyzed autolyzed, and also acid hydrolyzed yeast finding from 0.3 to 0.5 per cent of the yeast nitrogen as choline in different experiments. The above figures are in substantial agreement with this. It is of interest to note the relatively large proportion of the nitrogen of yeast extract which occurs as free choline. The proportion of this base is about three times as great as that found in the alfalfa plant (0.063 per cent) (2).

There was no indication of the presence of bases of the betaine type in yeast. If present at all they can form only a very small proportion of the total nitrogen.

Nicotinic acid was first discovered in nature in rice polishings by Suzuki, Shimamura, and Odake (6) and was subsequently identified by Funk (7) as a constituent of his "vitamin" fraction

TABLE II.

Yields of Choline and Nicotinic Acid Obtained from Yeast Extract Representing 6145 Gm. of Dry Yeast Containing 484.8 Gm. of Nitrogen.

	Amount.	N	Dry yeast.	N as N of dry yeast.	N as N of yeast extract.
	gm.	*gm.*	*per cent*	*per cent*	*per cent*
Choline	11.54	1.336	0.19	0.29	2.07
Nicotinic acid	1.13	0.1803	0.018	0.039	0.280

from yeast. So far as we can learn it has been found in no other materials. The proportion found by Funk was little more than a trace as he obtained less than half a gm. from 100 kilos of dry yeast. The figures given above indicate that nicotinic acid is a by no means insignificant constituent of yeast. Trigonelline, the betaine of nicotinic acid, is not an uncommon constituent of higher plants and has recently been found in an animal, *Arbatia pustulosa*, by Holtz, Kutscher, and Thielmann (8). Ackermann (9) found that nicotinic acid, fed to dogs, was excreted as trigonelline. It would seem, therefore, that the capacity to methylate nicotinic acid, possessed by many higher plants and by certain animals, is lacking in yeast. The significance of this may become more apparent when the physiological relations of the betaines are better understood.

EXPERIMENTAL.

An aliquot part of the choline fraction containing 0.2884 gm. of nitrogen (by Kjeldahl) was freed from reagents and employed for the fractional crystallization of the picrates. The solution was evaporated to a sirup, an equivalent of picric acid dissolved in alcohol was added, the solution heated to dissolve the precipitated picrate, and allowed to stand. A large crop of yellow needles melting at 241–244°C. separated. A second crop, obviously impure, was obtained on concentration and four more successive crops of material were likewise removed. The final mother liquor consisted of a few drops of oil. These six crops were put through a systematic recrystallization from absolute alcohol and thereby separated into needles of choline picrate melting at 247°, weighing 5.869 gm., and nicotinic acid picrate, separating in nodular aggregates of short prisms and weighing 0.465 gm. These were obtained in short rhombic prisms melting at 221° on recrystallizing from water. These quantities are equivalent to 0.2475 gm. of choline nitrogen and 0.0185 gm. of nicotinic acid nitrogen. Multiplied by 5.394, the factor for the aliquot used, they represent 1.336 gm. of choline and 0.0997 gm. of nicotinic acid nitrogen from the whole fraction of the yeast extract.

The choline picrate was recrystallized from absolute alcohol when it melted at 248–249° to a clear oil. Decomposition with evolution of gas began at about 270°. The nitrogen content was 16.8 per cent, theory for choline picrate 16.87 per cent. Yoshimura and Trier (10) report the uncorrected melting point of choline picrate at 240–241°. The above melting points were taken with a short stem Anschütz thermometer.

The nicotinic acid picrate contained 65.5 per cent picric acid (nitron method), theory 65.05 per cent, and, on recrystallizing, melted at 221–222° to a red oil which began to decompose at 250°. Funk gives the uncorrected melting point of this substance at 219°. The free acid was obtained from the picrate in colorless needles melting at 237–238° (Funk gives 235° uncorrected) and subliming into the cooler parts of the tube, when heated above the melting point, with little if any decomposition. The chloride melted at 272° with decomposition and the nitrate at 184–185°. Pictet and Rotschy give 185° (11). For further identification

nicotinic acid was prepared from nicotine following the directions of Pictet and Sussdorff (12). The free acid melted at 236° and when mixed with an equal amount of the free acid from yeast the melting point was likewise 236°. The picrate was prepared from this acid. It melted at 221–222° and when mixed with an equal amount of the picrate from yeast the melting point was unchanged.

The isolation of a further quantity of nicotinic acid as picrate from the filtrate from the mercuric chloride precipitate has already been described. This was equivalent to 0.0806 gm. of nicotinic acid nitrogen. Identity with the material isolated from the precipitate was demonstrated by a mixed melting point determination.

It is of some importance to note that about one-half of the nicotinic acid present was precipitated in acid solution by the mercuric chloride along with the choline.

SUMMARY.

A study of the nature of the simpler nitrogenous substances extracted from fresh brewers' yeast by hot water, using the methods previously employed in the study of alfalfa juice, is in progress. This paper deals with the basic substances not precipitated by Neuberg's reagent. These contained nitrogen equivalent to 3.35 per cent of the nitrogen of the yeast extract. When fractionated with mercuric chloride in acid solution, 2.41 per cent of the nitrogen of the yeast extract was found in the precipitate. Of this nitrogen about 86 per cent belongs to choline and 6 per cent to nicotinic acid. Further quantities of nicotinic acid were found in the filtrate from the mercuric chloride precipitate. Choline was found to account for 2.07 per cent of the nitrogen of the yeast extract and 0.29 per cent of the nitrogen of the dry yeast. Nicotinic acid accounts for 0.28 per cent of the nitrogen of the extract and 0.039 per cent of that of the dry yeast.

Bases of the betaine type, if present at all in yeast, can occur only in very small proportions.

Since the yeast extract was not subjected to hydrolysis these substances occur free in the cell solution to at least the extent given.

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THE PREPARATION OF ADENINE NUCLEOTIDE FROM TEA LEAVES.

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In 1885 Kossel (1) hydrolyzed some "nuclein" from pancreas with dilute sulfuric acid and obtained, beside guanine, xanthine, and hypoxanthine, a new base to which he gave the name adenine. Later (2) he found it present in other animal nucleins and in the nuclein from yeast. He made the assumption that it was a constituent of the cell nucleus and would probably be present in all extracts of animal and plant tissue. With this in mind he hydrolyzed a large quantity of commercial tea extract, which he knew to contain substituted purine bases, and found adenine present in considerable amount. Kossel's assumption that the adenine was linked up physiologically with the nucleic acid of the cell, as he thought the other purine bases were which he had previously found, was correct, but he made no attempt to isolate adenine in a combined form.

Krüger (3) repeated the work of Kossel with a slight modification of his method (4) and obtained large quantities of adenine. He likewise made no attempt to get the adenine in a combined form.

Adenine combined as adenine nucleotide was first isolated and obtained in crystalline form by Jones and Kennedy (5) by oxidation of yeast nucleic acid with potassium permanganate. It has since been prepared a number of times from a variety of sources, and its properties have been thoroughly studied.

The object of this research was to investigate the possibility of the isolation of adenine in the form of adenine nucleotide from an extract of tea leaves. With this aim in view the method of Steudel and Peiser (6) for hydrolysis of yeast nucleic acid was used to hydrolyze any combined nucleotides that might be present. The method consists simply in hydrolysis *at room temperature* with very dilute sodium hydroxide, subsequent acidification with acetic acid, and precipitation of the nucleotides as the lead salt with neutral lead acetate. The lead salts were decomposed with hydrogen sulfide and filtered. The filtrate was aerated and evaporated

to a small volume under diminished pressure at a low temperature. After further concentration to a thin syrup in a vacuum desiccator over sulfuric acid it was hardened by means of absolute alcohol. The alcohol removed all the gallic acid, which was present in large quantities, and also a great part of the coloring matter.

The matter of removing the tannins and pigments was difficult since they were precipitable both by lead acetate and brucine. They were finally removed, however, because of their relative solubility in absolute alcohol whereas the adenine nucleotide was insoluble.

The usual procedure of separation of the crude mixed nucleotides by means of the ammonium salts into two fractions with 50 per cent alcohol was followed. The adenine nucleotide remained in the filtrate which is ordinarily called the adenine fraction, but there was no trace of guanine found in the dark gray precipitate, which is designated as the guanine fraction when yeast nucleic acid is the material under investigation. It is interesting to note in this connection that neither Kossel (2) nor Krüger (3) reported guanine and made no effort to remove it in their preparation of adenine from the extract of tea leaves.

The adenine nucleotide obtained in this investigation was identical in its physical and chemical properties and in its chemical composition with the adenine nucleotide obtained by Jones and Kennedy (5) from yeast nucleic acid by oxidation with permanganate and by Jones and Abt (7) by hydrolysis with ammonia under pressure. It crystallized in long prismatic needles from a water solution and on heating to 110° it lost its water of crystallization and regained it on exposure to the air. It was unmistakably identified by the pentose color reactions, by the preparation and analysis of the characteristic crystalline adenine sulfate, and by the melting point and crystalline form of adenine picrate, which crystallized in long, pale yellow needles resembling matted hair. The amount of the crude nucleotide obtained before the preparation of the brucine salt would seem to indicate that it is present in a much larger quantity than has been found.

EXPERIMENTAL.

Extraction of Tea Leaves.—The cheapest grade of tea leaves that could be obtained was worked up in 2 pound lots. The leaves

were ground almost to a powder in a mill and suspended in 6 liters of 2½ per cent sodium hydroxide solution. This formed a thin paste and was allowed to stand for 24 hours at room temperature in order that extraction and hydrolysis might be as complete as possible at this temperature. It was then pressed out through folded cheese-cloth. The residue was washed once with a liter of distilled water and pressed out a second time. A dark brown liquid was obtained. It was made distinctly acid with acetic acid and a voluminous precipitate of insoluble material settled out immediately. It was filtered off and the filtrate, which was still dark brown, was used for the preparation of the crude nucleotide.

Preparation of the Crude Nucleotide.—The filtrate obtained above was heated up to 80–90° on a steam bath. A 25 per cent solution of lead acetate was added slowly to the hot solution until no further precipitate formed on addition of more lead acetate to a portion of the cooled, filtered liquid. 20 cc. of lead acetate were added in excess of the above requirement and the hot solution cooled, usually by allowing it to stand overnight. The lead acetate precipitate was filtered or centrifuged from the pale yellow liquid and washed three times by grinding with large volumes of cold distilled water. The washed precipitate was suspended in hot water and decomposed with hydrogen sulfide. The dark brown filtrate from the lead sulfide was freed from hydrogen sulfide by aeration and taken through the lead procedure a second time. After removal of the hydrogen sulfide the filtrate, which was still very dark, was concentrated, under reduced pressure at 40–50°, to a small volume and finally in a vacuum desiccator to a thin syrup. This syrup was hardened by pouring it into ten times its volume of absolute alcohol. The finely divided light brown precipitate was filtered off and washed twice with 100 cc. of absolute alcohol at each washing. When dry this precipitate varied in weight from 12 to 15 gm. At this point three lots were usually combined and about 40 gm. of the crude material were dissolved in 400 cc. of hot water, let stand overnight, and filtered from a small amount of insoluble material. The filtrate, which was still dark brown, was made faintly alkaline to litmus with ammonia and treated with 450 cc. of absolute alcohol. The dark gray gelatinous precipitate was filtered off and washed with 50

per cent alcohol. The combined washings and filtrate were made faintly acid with acetic acid, treated with an equal volume of boiling water, and taken through the usual lead process. The filtrate from the lead sulfide, after aeration, was concentrated by the usual procedure and hardened with absolute alcohol. The yield of hardened material at this point was 4 to 5 gm. The hardened material when dry was nearly white but its water solution was highly colored.

Preparation and Purification of the Brucine Salt.—The crude nucleotide obtained above was dissolved in the smallest possible amount of hot water (20 to 25 cc.) and neutralized with a hot saturated solution of brucine in alcohol. This gave 12 to 14 gm. of a colored brucine salt which did not lose its color on recrystallization. It was then decomposed by suspension in hot water and treatment with ammonia, cooled, and filtered. The filtrate was extracted three times with chloroform which removed the last traces of brucine. The solution was made faintly acid with acetic acid and taken through the lead process again. The filtrate from the lead sulfide was still slightly colored. After aeration it was concentrated in a vacuum desiccator over sulfuric acid and hardened with absolute alcohol. The brucine salt was made from the hardened material as before and after one recrystallization from 35 per cent alcohol was almost pure white and gave the following analysis.

0.2830 gm. required 10.2 cc. of standard acid (1 cc. = 0.002876 N).

	Required for brucine salt of adenine nucleotide.	Found.
N	10.0	10.36

The above brucine salt after six recrystallizations from 35 per cent alcohol was also analyzed.

0.2250 gm. required 7.87 cc. of standard acid (1 cc. = 0.002876 N).
0.2568 " " 8.95 " " " " (1 " = 0.002876 ").

	Required for brucine salt of adenine nucleotide.	Found.	
		I	II
N	10.0	10.06	10.02

Preparation of the Crystalline Adenine Nucleotide from the Brucine Salt.—The Brucine salt obtained by the method described above, after one recrystallization from 35 per cent alcohol, was suspended in hot water, decomposed with an excess of ammonia, cooled, and filtered. The filtrate was extracted three times with chloroform, made slightly acid with acetic acid, and carried through the lead process. The filtrate from the lead sulfide was practically colorless. After aeration it was concentrated in a vacuum desiccator to a small volume and allowed to stand for several days. Crystalline adenine nucleotide separated in long prismatic needles. The yield varied from 0.3 to 0.5 gm. from 6 pounds of tea.

I. 0.2030 gm. required 13.43 cc. of standard acid (1 cc. = 0.002876 N).
II. 0.2138 " " 14.26 " " " " (1 " = 0.002876 ").
III. 0.2776 " lost 0.0130 gm. at 110° and regained 0.0129 gm. on exposure to air.
IV. 0.1832 gm. gave 0.1222 gm. of $MgNH_4PO_4 \cdot 6H_2O$.
V. 0.1765 " " 0.1170 " " $MgNH_4PO_4 \cdot 6H_2O$.

		Calculated for $C_{10}H_{14}N_5PO_7 \cdot H_2O$.	Found.				
			I	II	III	IV	V
N		19.18	19.02	19.18			
H_2O	Lost	4.93			4.68		
	Regained				4.68		
P		8.49				8.43	8.37

The mother liquors from the combined brucine salts from 12 pounds of tea leaves were added to the filtrates from the first crystallization of the brucine salts and evaporated to a small volume, cooled, and filtered. The crystalline precipitate of crude brucine salts was suspended in warm water and treated with an excess of ammonia. The mixture was cooled and filtered from brucine and the filtrate was extracted three times with chloroform. It was then evaporated to dryness on a water bath, taken up in twenty-five times its weight of 7 per cent sulfuric acid, filtered from a small insoluble residue, and hydrolyzed by immersing in a boiling water bath for 1 hour. The hot sulfuric acid solution was neutralized with ammonia and enough excess was added to make the solution approximately 2 per cent. This was allowed to stand

overnight and a small amount of dark brown residue filtered off. The highly colored filtrate was treated with a solution of silver nitrate and a heavy gelatinous precipitate of silver adenine was immediately formed. This precipitate was filtered off, washed thoroughly, suspended in warm water, and a slight excess of hydrochloric acid was added over the amount required to precipitate the silver as silver chloride. The filtrate from the silver chloride was concentrated to a paste on a water bath, taken up in water, and evaporated to a paste a second time. This was repeated once more with water and once with alcohol. The residue was taken up in 100 cc. of water and 5 cc. of 10 per cent sulfuric acid, and the solution decolorized with a vegetable charcoal. This sulfuric acid solution was concentrated to about 2 cc., taken up in 20 cc. of water, and evaporated a second time to a small volume to remove the last traces of hydrochloric acid which can be tested for with silver nitrate. 5 cc. of 10 per cent sulfuric acid and 10 cc. of water were added and the solution allowed to cool in an ice box overnight. Beautiful crystals of adenine sulfate separated. They were recrystallized once from hot water and analyzed. The adenine sulfate is so insoluble in cold water that the recrystallization may be repeated as many times as necessary without appreciable loss of material. This is a modification of the method of Jones and Perkins (8).

0.1832 gm. required 22.02 cc. of standard acid (1 cc. = 0.002876 N).
0.1543 " " 18.58 " " " " (1 " = 0.002876 ").

	Required for $(C_5H_5N_5)_2H_2SO_4 \cdot 2H_2O$.	Found.	
		I	II
N.....	34.65	34.55	34.63

Preparation of Adenine Picrate.—A small amount of the above adenine sulfate was dissolved in hot water and treated with an excess of a saturated solution of picric acid. A canary yellow precipitate formed immediately which went into solution on heating. When cool the adenine picrate separated in long intertwined needles resembling matted hair. When the melting point was taken according to the method described by Vickery and

Leavenworth (9), it gave the characteristic darkening at 285° and melted sharply at 296°, uncorrected, using a long thermometer.

SUMMARY.

A crystalline substance has been obtained from tea leaves which is identical with the adenine nucleotide obtainable from yeast nucleic acid.

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A STUDY BY MEANS OF ULTRAFILTRATION OF THE CONDITION OF SEVERAL INORGANIC CONSTITUENTS OF BLOOD SERUM IN DISEASE.

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MacCallum and Voegtlin (1) first demonstrated that the calcium concentration of the blood in tetany parathyroipriva is reduced to about 50 per cent (or more) of the normal. They also showed that the administration of calcium chloride to animals will relieve their convulsions and maintain them free from symptoms as long as the calcium chloride is continued. These facts have been confirmed by numerous investigators. Salvesen (2) by employing more accurate methods for the determination of the inorganic constituents of blood has recently found that the early contention of MacCallum and Voegtlin as regards the rôle played in this condition by the calcium is entirely correct. He was able to keep parathyroidectomized dogs alive on milk and calcium chloride for as long as 2 years. In this connection he brought out a most interesting fact, namely that although some animals may remain free from convulsions, yet their blood calcium concentration does not rise above the so called tetany level.

Howland and Marriott (3) have shown that in infantile tetany there is a marked reduction in the calcium concentration in the serum and that the convulsions can be controlled by the administration of calcium salts. These observations have been confirmed by Kramer, Tisdall, and Howland and by others. Grant and Goldman (4) have reported, however, that when tetany occurs following hyperpnea the calcium concentration of the serum remains normal and may even be somewhat increased. Freudenberg and György (5) state that the symptoms of tetany in infants may be suppressed when acid-producing salts such as ammonium

chloride and ammonium phosphate are administered although the calcium concentration of the serum may remain below the level at which tetany commonly occurs. The administration of ammonium chloride according to McCann (6) and others relieves the symptoms of gastric tetany without producing any change in the calcium concentration of the serum. Marriott and Howland (7) and more recently Salvesen and Linder (8) have reported low calcium findings in the serum of patients with nephritis who presented no symptoms of tetany. Freudenberg and György attempted to harmonize these apparently discordant findings by assuming the existence of a reduction of the ionized calcium in all of these conditions in which tetany is a common symptom. To establish the correctness of their hypothesis it is necessary to measure the calcium ion concentration of the serum.

We have no method for measuring the actual calcium ion concentration of the serum or plasma and must therefore resort to indirect means of determining this value. Several attempts have been made in this direction. Trendelenburg and Goebel (9) showed that the frog's heart when perfused with a solution of the ash of the serum of a dog in tetany beats less forcibly than when the ash of normal serum is used. This is probably due merely to the difference in total calcium in the two sera and is not a measure of the ionic calcium in the original serum. Rona (10) and his coworkers used compensation dialysis as did von Meysenbug and Pappenheimer. We have chosen the method of ultrafiltration through collodion bags under moderate pressure as described by Neuhausen and Pincus.

Neuhausen and Pincus (11) have shown that filtrates of calcium chloride solutions contain the same concentration of calcium as do the original solutions and that the concentration of calcium in the filtrate remains constant in consecutive samples of filtrate. When normal serum is filtered the calcium concentration of the filtrate is only 50 per cent to 60 per cent of that of the original serum. If the reaction of the serum is changed to that approximating the isoelectric point of the serum proteins calcium appears in the filtrate in about the same concentration as in the original serum (see Table I). This fact we believe indicates that ultrafiltration is a method for determining the relation that exists between the free and combined calcium in the serum.

The fact that all the calcium may be precipitated as calcium oxalate merely by adding an excess of ammonium oxalate to serum would speak for an equilibrium between bound and free calcium which tends to go to completion in one direction; namely, the formation of free calcium ions when any reagent is added which removes calcium ions as soon as they are formed. If it be granted that calcium which does not filter through collodion is bound to protein and is practically unionized and that all of the calcium which does filter is completely ionized (complete dissociation of strong electrolytes in dilute solution) then the concentration of calcium in the filtrate is a measure of ionized calcium. Even if these assumptions are not granted our results indicate a difference between the two calcium fractions in disease.

By employing the method of ultrafiltration we have studied the

TABLE I.

Ultrafiltration of Sera of Different Reactions.

pH	Total Ca.	"Free" Ca.	Residue Ca.
7.1	9.4	5.0	10.1
4.7	8.3	8.1	8.6
4.7	9.0	8.9	9.3

These results indicate that at the isoelectric point of the serum proteins the concentration of calcium in the ultrafiltrate is practically identical with that of the original serum.

relationship between the "bound" or unfiltrable calcium and the "free" or filtrable calcium in patients with chronic nephritis and infantile tetany and in the serum of completely parathyroidectomized dogs—some of which were exposed to radiation from the mercury vapor quartz lamp, others treated with milk and calcium chloride. We have also determined the total protein concentrations in the blood of some of these animals.

Methods.

The parathyroids were removed under ether anesthesia. Blood samples for study were obtained before operation. Serum was collected in the usual manner. Samples of blood were again obtained when the animal showed either signs of beginning tetany, *i.e.* twitching or tremors, or when pronounced convulsions were

present. Whenever possible samples of blood were obtained for the purpose of chemical analysis at regular intervals after the onset of symptoms.

Two series of animals were studied. One group was treated with the mercury vapor quartz lamp beginning with 5 minutes exposure and increasing this gradually until a daily exposure of 30 minutes was given. The distance was about 20 inches and the strength of current about 4 amperes. The second group was treated with milk and calcium chloride.

In our cases of infantile tetany blood was obtained both during

TABLE II.

Chemical Determinations on the Blood Serum of Dogs before and after Parathyroidectomy.

Date.	Diagnosis.	Serum.		Ultrafiltrate.		Total protein.
		Ca	P	Ca	P	
		Dog K 30.				
1924						
Apr. 29	Normal.	11.8	6.2	6.1	6.4	7.2
May 4	Tremors.	7.4	7.2	2.8		7.1
" 13	Convulsions.	6.0	12.6	2.1		7.8
		Dog K 32.				
May 20	Normal.	10.6	5.5	5.6		7.2
" 23	Convulsions.	5.8	9.1	3.1		8.0

Table II shows a marked decrease of the serum calcium concentration and a rise in the inorganic phosphorus concentration after parathyroidectomy. The protein concentration of the serum remains unaltered.

active tetany and several weeks later after active therapy with calcium chloride and cod liver oil had been instituted.

Blood from severe forms of chronic nephritis was obtained in one instance from a case in uremic convulsions. The non-protein nitrogen was above 100 mg. for 100 cc. of blood in all cases studied. In every instance the patient came to autopsy and the clinical diagnosis was verified.

The calcium was determined by the method described by Kramer and Tisdall (12). Phosphorus determinations were done by the Briggs modification of the colorimetric method of Bell and Doisy (13).

TABLE III.

Chemical Determinations on the Blood Serum of Dogs before and after Parathyroidectomy and after Prolonged Treatment with Mercury Quartz Lamp Radiation.

Dog.	Date.	Diagnosis.	Serum.		Ultrafiltrate.		Protein.
			Ca	P	Ca	P	
	1924						
K 16	Jan. 5	Normal.	9.2	6.1	5.6	6.6	7.0
K 16	" 18	Tetany.	5.6	10.1	3.5	12.2	7.3
K 16	" 25	Radiated.	5.7	11.2	3.1	12.5	7.7
K 16	Feb. 3	"	5.4	10.8	3.2	11.7	7.5
K 19	Jan. 18	Normal.	10.6	4.8	5.5	5.7	6.8
K 19	" 19	Tetany.	6.3	6.8	3.8	6.9	7.7
K 19	" 24	Radiated.	5.9	5.2	2.2	6.0	7.2
K 19	Feb. 1	"	5.6	5.4	2.3	6.1	7.5
K 19	" 4	"	5.7	3.2	2.2	4.0	7.1
K 22	Jan. 20	Normal.	10.7	7.4	5.6	7.2	7.3
K 22	" 22	Tetany.	6.8	10.8	3.6	12.0	8.2
K 22	" 28	Radiated.	5.5	12.2	2.3	13.3	8.0
K 22	Feb. 7	"	5.6	12.6	2.2	13.8	8.4
K 22	" 14	"	6.0	12.1	2.7	13.3	8.1
K 22	" 23	"	5.8	10.7	2.2	12.5	7.7
K 24	Feb. 1	Normal.	9.9	3.3	5.6	3.5	7.4
K 24	" 2	Tetany.	6.0	6.8	3.2	6.5	7.1
K 24	" 9	Radiated.	4.9	8.3	2.2	8.6	
K 24	" 16	"	4.1	6.2	2.1	7.5	7.7
K 24	" 23	"	4.6	7.0	2.0	8.6	6.3
K 24	Mar. 1	"	4.5	6.8	2.0	8.1	7.0
K 24	" 9	"	4.9	6.0	2.1	7.3	7.2
K 27	Jan. 26	Normal.	10.2	5.2	5.4	6.0	7.1
K 27	" 28	Tetany.	6.8	8.3	3.1	9.1	7.6
K 27	Feb. 6	Radiated.	5.9	9.0	2.8	10.1	7.9
K 27	" 14	"	5.2	8.6	2.2	9.7	
K 27	" 20	"	5.3	8.5	2.2	9.0	6.8
K 27	" 29	"	5.5	9.2	2.2	9.8	7.1
K 27	Mar. 6	"	5.2	8.1	2.1	9.3	6.6

Total protein was determined by the refractometer method using Neuhausen and Rioch's (14) constants for the refractive indices of albumin, globulin, and the protein-free serum.

Results.

Tetany Following Parathyroidectomy.

In the normal animal (the dog in this instance) we found that the free calcium varies between 50 per cent and 60 per cent of the

TABLE IV.

Chemical Determinations on the Blood Serum of Patients with Infantile Tetany before and after Treatment.

Case.		Serum.		Ultrafiltrate.		Protein.
		Ca	P	Ca	P	
H. C., age 7 mos.	Active tetany.*	6.4	5.2	2.2	5.6	7.4
	Complete recovery.†	8.5	3.1	5.1	3.3	7.5
T. J., age 8 mos.	Active tetany.	5.8	6.0	2.0	5.2	7.1
	Complete recovery.	8.2	2.8	5.0	3.0	7.2
M. W., age 10 mos.	Active tetany.	6.1	6.2	2.2	6.4	6.8
	Complete recovery.	8.7	3.0	5.3	3.2	7.0

* Patients had all the classical signs of infantile tetany; convulsions carpopedal spasm, positive Chvostek and Erb signs.

† Electrical reactions became normal, Chovstek sign could not be elicited, and there were no other clinical evidences of tetany.

TABLE V.

Chemical Determinations on the Blood Serum of Patients with Chronic Nephritis.

Case.	Non-protein N.	Creatinine.	Serum.		Ultrafiltrate.		Protein.
			Ca	P	Ca	P	
A. S.	130	2.2	7.4	12.6	4.8	13.0	7.6
H. W.	96	1.5	6.8	10.5	4.6	11.0	7.7
B. G.	310	3.0	6.6	12.8	4.6	13.0	7.9
F. C.	261	2.4	6.1	14.0	4.3	14.7	8.1
J. M.*	190	2.5	6.3	15.6	2.8	15.8	7.5

* Patient had uremic convulsions.

total calcium concentration of the serum. The inorganic phosphorus we found to be entirely free, *i.e.* completely diffusible. Both of these results are in agreement with those obtained by Cushny (15), Rona and Takahashi, and Neuhausen and Pincus.

In the parathyroipriva tetany the free calcium of the serum gradually dropped to 50 or 40 per cent, or even to 30 per cent of the normal, the total calcium dropping in the same ratio. Exposure to ultra-violet light failed to raise the total calcium or the free calcium concentration but did seem to prolong the life of the animals. The inorganic phosphorus concentration was increased in every instance. Often an increase of 100 per cent was observed. This is in agreement with the findings of Greenwald (16) and of Salvesen. The total protein concentration varied slightly. (See Tables II and III.)

Infantile Tetany.

With infantile tetany the filtrable calcium was markedly decreased. Following treatment this component increased rapidly and reached the normal level when the total calcium was still slightly reduced. (See Table IV.)

Chronic Nephritis.

With a moderate decrease of the total calcium concentration the decrease was reflected entirely in the bound calcium. The filtrable fraction remained normal. (See Table V.)

DISCUSSION.

Von Meysenbug (17) and his coworkers, employing the method of compensatory dialysis, found no change in the diffusible calcium in parathyroipriva tetany. Cruickshank (18) claims that, employing this method, he obtained a diffusible calcium in tetany which amounts to about 94 per cent of the total, while we, by employing the method of ultrafiltration, have arrived at a widely different conclusion as to the amount of diffusible or filtrable calcium. We found in tetany the filtrable calcium to be about 30 per cent of that found in normal serum.

Our results with the various types of tetany which we have studied indicate that whenever active tetany is present there is a profound decrease in the free calcium. A low calcium concentration without tetany is possible (as in nephritis) but here the free calcium is practically unchanged. When cure was effected in the cases of infantile tetany the free calcium reached its normal level

(5 mg. per 100 cc. of serum) before the total calcium came up to the normal level of 10 mg. per 100 cc. of serum.

Our inorganic phosphorus findings are in accord with those obtained by all the other investigators. The concentration is always increased after parathyroidectomy, sometimes being 100 per cent higher than in the normal serum.

Our cases of chronic nephritis showed a low calcium concentration in the serum but the free calcium was normal in all but one patient. This patient was in uremic convulsions when the blood was taken for analysis. The phosphorus (inorganic) was high in every instance. This is in agreement with results obtained by Howland and Marriott.

Salvesen and Linder maintain that on a diet containing at least 500 cc. of milk per day, parathyroidectomized dogs may be kept alive and free of symptoms for a long period of time. We have not met with success in attempting to repeat this. We were, however, able to maintain dogs alive over a moderately long period of time (6 to 8 weeks) by irradiating them with the mercury vapor quartz lamp. In these dogs we found that the calcium reaches a certain minimal concentration which is maintained for a long time with freedom from convulsive seizures. The dogs, however, become very asthenic, lose their appetite, and die.

CONCLUSIONS.

1. In tetany, both infantile and experimental, there is a marked decrease in the "free" calcium of the serum.

2. In chronic nephritis, uncomplicated by uremic convulsions, the free calcium is normal. In one patient with convulsions it was definitely reduced.

3. The phosphorus (inorganic) is invariably high in parathyroipriva tetany and in severe chronic nephritis with or without uremic convulsions.

4. The total protein concentration of the serum in experimental and infantile tetany is about normal.

5. Ultrafiltration of the serum at a reaction of pH 4.7 shows that at this reaction the calcium is entirely filtrable.

6. The failure of the calcium concentration of the serum in tetany parathyroipriva to increase after irradiation with the mercury vapor quartz lamp as it does in patients with infantile

tetany makes it highly probable that infantile tetany is not a form of parathyroid tetany and this assumption is strengthened by the absence of any increase of the inorganic phosphorus of the serum of children with tetany comparable to that found in the serum of dogs after parathyroidectomy.

7. The fact that the free calcium of the serum may remain constant in patients with chronic nephritis even when the total calcium is definitely reduced, serves to explain why such patients do not develop convulsions.

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THE OXIDATION-REDUCTION POTENTIALS OF 2-OXYDIHYDROINDOLE-3-PROPIONIC ACID AND SOME OF ITS HALOGEN DERIVATIVES.

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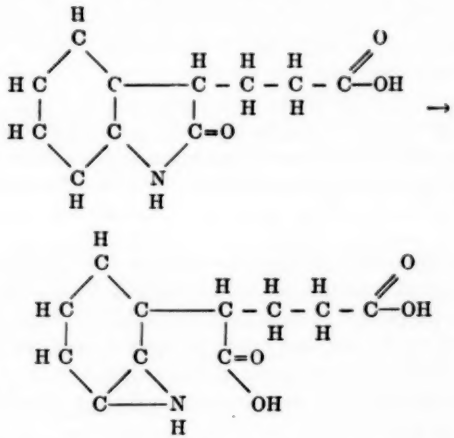
During the preparation of 2-oxydihydroindole-3-propionic acid, it was found that atmospheric oxygen will remove two atoms of hydrogen from this indole derivative and form a bond from carbon 7 to the nitrogen (6). It was then found that this lactam will also reduce some dyes such as dibromo and other indophenols. Since



the work leading to the synthesis of thyroxin indicated that this ability to reduce and oxidize resides not only in 2-oxydihydroin-

dole-3-propionic acid but is also a property of thyroxin and closely related compounds (5), it was decided to undertake the study of the oxidation-reduction potentials of this series of compounds.

Experiments were made first to show whether 2-oxydihydroindole-3-propionic acid reacted according to the usual equation which relates potential to the percentages of the oxidized and reduced forms present in the solution (1). When this compound or its halogen derivatives is added to a solution buffered to pH 7.4 and the solution is allowed to stand for from 5 minutes to 7

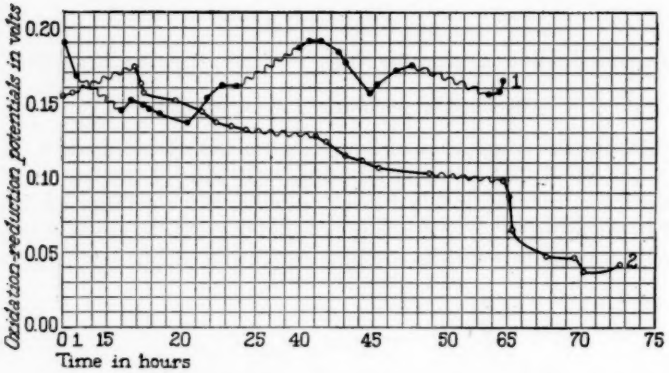


FIG. 1. Two solutions of diiodo-2-oxydihydroindole-3-propionic acid buffered to pH 7.4 gave the following oxidation-reduction potentials at the times indicated in the figure. The results show the fluctuations and wide divergencies in the potentials of two solutions prepared in an identical manner.

days, measurements of oxidation-reduction potentials will give a variable series of readings characterized only by fluctuations and drifts in the potentials (Fig. 1). If the oxidized form of this lactam is added to the reduced form, no more consistent results are obtained. If the concentration of the reduced form is changed, it does not necessarily mean that the reducing potential of the system is altered.

Since different platinum electrodes gave varying results, it seemed possible that the platinum electrode failed in some in-

stances to record the actual reducing potential of the system and this suggested the use of oxidizing dyes in order to determine by color reaction the approximate reducing power of the solutions. For this purpose, certain derivatives of indophenol were used, principally 2,6-dibromoindophenol (4). By the use of this dye it became evident that the platinum electrodes were registering the correct reducing potential of the system. When dibromoindophenol is added to 2-oxydihyroindole-3-propionic acid, the blue color of the dye is at first undiminished. After standing a short time, however, the reducing potential of the system increases and at the same time the blue color of the dye begins to fade. After several hours, the dibromoindophenol is completely reduced and the reducing potential of the system gives a corresponding value. The addition of dibromoindophenol to the completely reduced form of 2-oxydihydroindole-3-propionic acid will produce any desired percentages of oxidized and reduced forms of both 2-oxydihydroindole-3-propionic acid and of dibromoindophenol. The true oxidation-reduction potential of such a mixture can be determined by the platinum electrode, since Clark has shown that dibromoindophenol in its oxidized and reduced forms affects the platinum electrode in the usual manner (4).

A series of experiments was therefore carried out in which the sodium salt of the reduced form of the lactam was dissolved in a solution of pH 7.4, heavily buffered with phosphate mixture. To this was added a dilute solution of 2,6-dibromoindophenol. The value of the reducing potential before the addition of the indophenol was indefinite. In some cases the reading was the same as that of the buffer solution itself. In other cases, a slightly higher reducing potential was present. In still others a marked reducing potential was developed before the addition of any dibromoindophenol; but, regardless of the reading preceding the addition of the dye, the solutions all manifested oxidizing potentials which were remarkably consistent following the addition of a small amount of the dye. The value was approximately 0.25 volts. It was then found that as the dibromoindophenol was reduced, the reducing potentials increased and finally surpassed the reading obtained before the addition of the oxidizing agent (Fig. 2).

A paradoxical condition therefore resulted in these solutions. An oxidizing agent, dibromophenol, produced a reducing potential

higher than was originally present. Not only was this result confirmed many times, but it was shown that after a second addition of dibromoindophenol the reducing value was changed temporarily to the oxidizing potential of the new concentration ratio of reduced and oxidized dibromoindophenol present, followed, after a few hours' standing, by the former reducing potential and the complete reduction of the second portion of the dibromoindophenol. This action, following the addition of dibromoindophenol, could be repeated any desired number of times provided sufficient

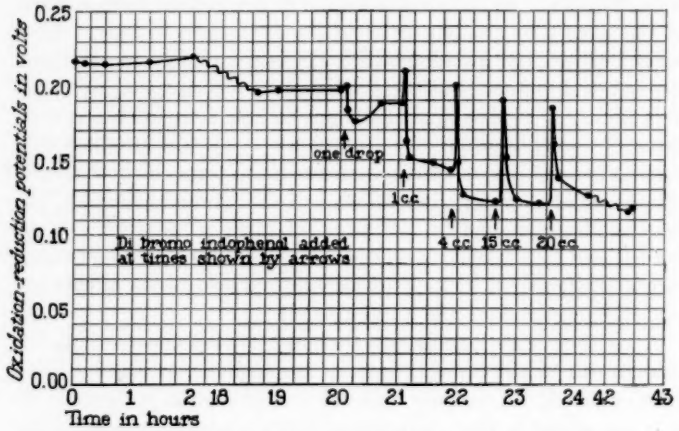


FIG. 2. Diiodo-2-oxydihydroindole-3-propionic acid in a solution buffered to pH 7.4 reacts with dibromoindophenol, giving first a decrease and then an increase in the value of the oxidation-reduction potentials. This same reaction is brought about by successive additions of the oxidizing agent.

of the reduced form of the 2-oxydihydroindole-3-propionic acid was present in the solution. These findings may be summarized as follows: Both the fully reduced and fully oxidized forms of this lactam have very little effect on the platinum electrode. The reduced form has little immediate action on dibromoindophenol. In the presence of this oxidizing compound, however, an intermediate substance is developed which has marked reducing power. The concentration of this intermediate form is at all times low. This is shown by the fact that after 2-oxydihydroindole-3-pro-

pionic acid has been partially oxidized with dibromoindophenol and a high reducing potential has been produced, only 0.5 cc. of N/50 solution of dibromoindophenol is required to destroy completely the high reducing potential (Fig. 2).

It having been shown that 2-oxydihydroindole-3-propionic acid can be oxidized with dibromoindophenol, it became of interest to determine the nature of the oxidation of this lactam; whether it

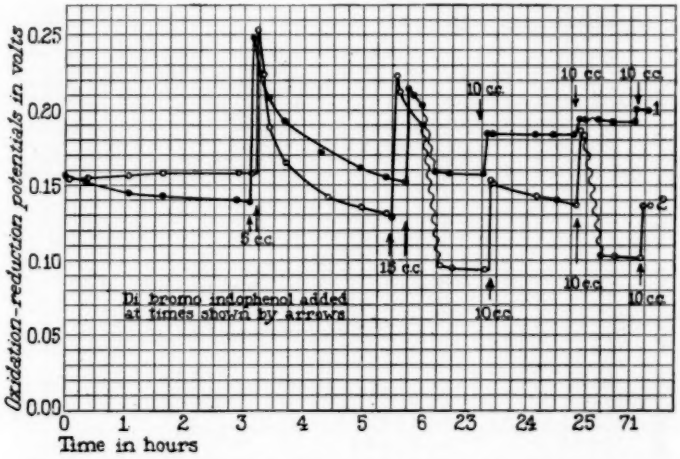


FIG. 3. The concentration of 2-oxydihydroindole-3-propionic acid affects the form of the curve after the addition of dibromoindophenol. In Solution 1 the dibromoindophenol oxidized the lactam, resulting in a much lower equilibrium point than in Solution 2 which still contained unoxidized lactam. The same amount of dibromoindophenol was added to each solution.

was autocatalytic or followed the monomolecular equation. It was found that the slope of the curve representing the increase in the reduction potential following the addition of dibromoindophenol was independent, within certain limits, of the amount of dibromoindophenol added. 1, 2, and 5 cc. of dibromoindophenol solution produced oxidations, the curves of which were similar. The slope of the curve representing the oxidation, however, depends on the amount of dibromoindophenol which has been added

previous to these additions. As the concentration of the dye increases in the solution, the rate of reduction of dibromoindophenol becomes less rapid. Although the potential which is established in the solution is due to the percentages of the reduced and oxidized forms of dibromoindophenol, this value depends on the amount of the reduced form of the lactam which is the source of the reducing activity. It is readily shown that, as more and

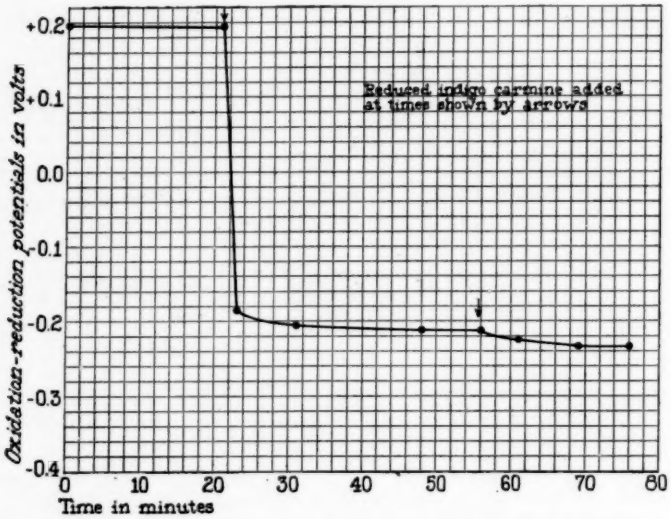


FIG. 4. The oxidized form of 4,6-dibromo-2-oxydihydroindole-3-propionic acid is not reduced in the presence of reduced indigo carmine, although a high reducing potential is present. No poising action of the oxidized lactam could be demonstrated.

more of the lactam is oxidized, the speed of reduction diminishes (Fig. 3).

When the results were used in an equation of the first order, a fair approximation was obtained for the value of the constant, the first part of the reaction being neglected. Moreover, when the results were used to test for reactions of the second or higher orders, no agreement at all was obtained.

The reaction apparently takes place in two stages. The first is

the formation of an addition compound between the oxidizing agent and the lactam, probably through the atom of nitrogen. This addition product then rearranges with the elimination of two atoms of hydrogen resulting in reduction of the dye and the production of the oxidized form of the lactam which has no effect on the platinum electrode. The active intermediate compound must be the addition product between the dye and the amine group

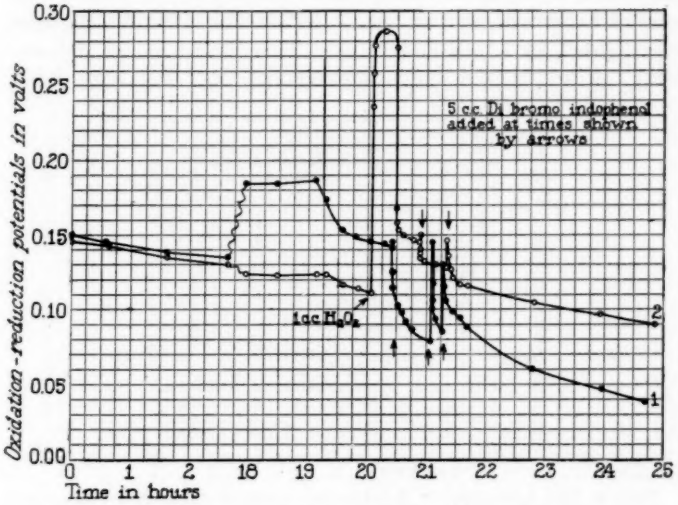


FIG. 5. In the presence of hydrogen dioxide the velocity of reaction between 4,6-diiodo-2-oxydihydroindole-3-propionic acid and dibromoindophenol is greatly diminished. Curve 1 shows the results of the addition of dibromoindophenol to the lactam. Curve 2 shows the effect on the potentials of 1 cc. of N/50 hydrogen dioxide, and then the result of the addition of the same amounts of dibromoindophenol, which were used with Curve 1.

of the lactam. This intermediate and unstable compound does not produce any definite reducing potential, but the value varies according to the dye used and to the total concentration of the oxidized and reduced forms of the dye in the solution.

In order to bring out the stability of the fully oxidized form of this lactam it was dissolved in a buffered solution of pH 7.4, and to

this, reduced indigo carmine or reduced indigo was added (7). Although the indigo derivatives developed high reducing potentials within the electrode chamber which was recorded by the platinum electrode, in no case was any poising action shown and the oxidized form of 2-oxydihydroindole-3-propionic acid remained unreduced (Fig. 4).

The intensity of reduction of the reduced form is established quantitatively by the reducing potentials determined, but it was of interest to check these readings by the addition of some of the dyes investigated by Clark and Cohen. This lactam in its reduced form can reduce not only dibromoindophenol but also naph-

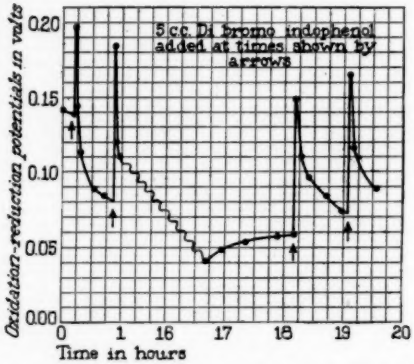


FIG. 6. The oxidation of 4,6-dibromo-2-oxydihydroindole-3-propionic acid is more rapid than is the oxidation of the unhalogenated lactam.

tholdichloroindophenol (3). It cannot, however, reduce indigo carmine.

After establishing the fact that the potential developed in the electrode chamber is dependent on the presence of an addition product between the dye and the lactam, it became apparent why the reduction potentials, determined without the addition of dibromoindophenol, gave such variable results. The lactam is not only sensitive to dibromoindophenol but will react with other compounds. Merely heating the solution of the diiodo lactam will produce a large increase in reducing potential. The variable amounts of the activated form of the lactam account for the

changes and drifting in the reducing potentials when the solutions were allowed to stand for several hours or days.

The delicately balanced condition of this lactam was brought out by its interaction with hydrogen dioxide. 1 cc. of N/50 hydrogen dioxide added to a solution containing thirty times the equivalent amount of the sodium salt of 4,6-diiodo-2-oxy-2,3 dihydroindole-3-propionic acid produced a drop in the reducing potential, but the value was not as low as that produced by adding hydrogen dioxide to the buffer alone. It was then found that the

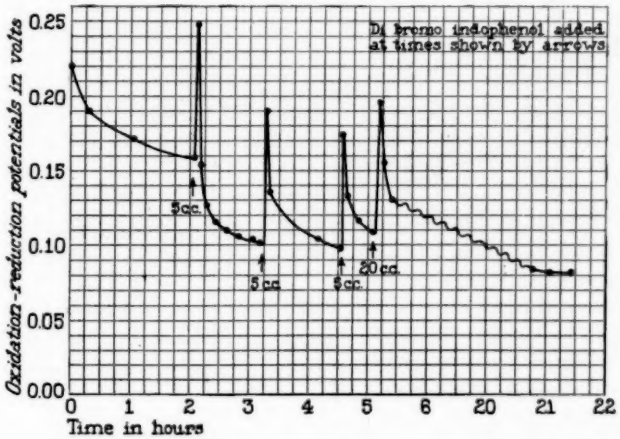


FIG. 7. The oxidation of 6-monoiodo-2-oxydihydroindole-3-propionic acid with dibromoindophenol is similar to the unhalogenated lactam, but more rapid.

interaction between the lactam and dibromoindophenol was reduced to an extremely slow rate and oxidation of the lactam was almost completely prevented. No explanation of this action can be given but it is evidence for the theory that some type of addition product is formed between the lactam and hydrogen dioxide (Fig. 5).

After the properties of 2-oxydihydroindole-3-propionic acid had been established, it became of great interest to determine the effect of adding halogen to the molecule. For this purpose, the

sodium salt of the 4,6-dibromo derivative was added to the buffer and the potentials were determined before and after the addition of dibromoindophenol. The results were similar to those obtained with the unhalogenated lactam. The maximal reducing power was about the same as that of the unhalogenated lactam, but it was found that the speed of reaction of the dibromo derivative was greater (Fig. 6). This greater speed of reaction, however, was the only material difference between the two compounds.

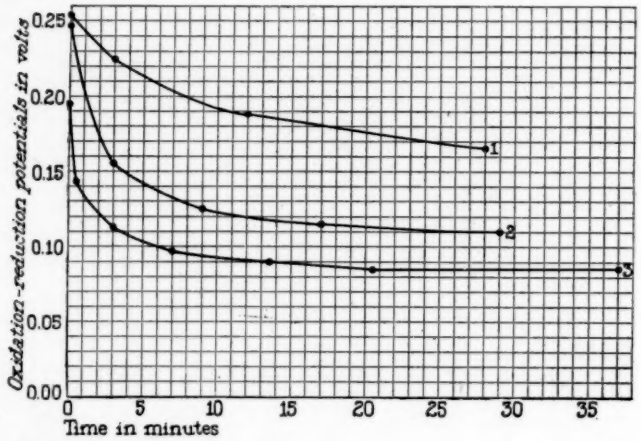


FIG. 8. The addition of halogen to 2-oxydihydroindole-3-propionic acid increases the velocity of reaction with dibromoindophenol. Curve 1 gives the velocity of oxidation of the unhalogenated lactam. Curve 2 is the velocity of oxidation of the monoiodo derivative, and Curve 3, the velocity of oxidation of the dibromo derivative of this lactam. The results are charted from Figs. 3, 6, and 7.

The 6-monoiodo and the 4,6-diiodo derivatives behaved in a manner similar to the dibromo lactam except that with the diiodo compound the speed of reaction was still greater (Figs. 7 and 8). It was also found possible to allow the sodium salt of the 4,6-diiodo derivative to remain many hours in the solution buffered to pH 7.4 in contact with the platinum electrode without the increase in reducing potential being manifested that had been produced with dibromoindophenol (Fig. 2). However, the reducing

potential developed after addition of the dye was not significantly different from that produced by the addition of the dye to the unhalogenated lactam.

Apparatus and Material.

The apparatus used for this work was similar to that devised by Clark except in one important detail (3). This consists in an arrangement which permitted the removal of the electrode chamber at any time from the thermostat without interruption of the flow of nitrogen. The complete apparatus consisted of a thermostat maintained at 30° ± 0.02°. Above this a frame was arranged supporting four electrode chambers fastened to four slides, which could be raised and lowered about 14 inches. A mercury seal allowed the continual bubbling of oxygen-free nitrogen through the solution irrespective of the position of the electrode chamber. Instead of the calomel half-cell communicating directly with the contents of the electrode chamber, a salt bridge was arranged which completed the circuit between the contents of the electrode chamber and a beaker of saturated potassium chloride into which the mercury half-cell dipped. The potentiometer was a Leeds and Northrup type K, and the galvanometer was a Leeds and Northrup high sensitivity type. Potential differences of less than 1 microvolt could be detected, but no attempt was made to read closer than $\frac{1}{10}$ millivolt. The single electrode potential of the normal hydrogen electrode being designated as zero, the reference standard for this work was a hydrogen electrode in M/20 potassium acid phthalate at 30° which gave a potential difference against saturated calomel electrodes very close to Clark's value of 0.4827 volts (2). Accordingly the value of +0.244 was assumed for the saturated calomel electrodes used. The potentials as read were rounded out to the nearest millivolt and subtracted from +0.244 to give the values recorded.

The nitrogen was purified by passing through three quartz tubes, 2.5 cm. in diameter and 90 cm. long, arranged in series and filled with copper made from copper oxide wire by reduction with hydrogen. The quartz tubes were wound with nichrome resistance wire and were heated to a dull red. The nitrogen passed from one tube to another through Pyrex glass tubing which was sealed into the quartz with De Khotinsky's cement. These three

quartz tubes were able to reduce all traces of oxygen even when the nitrogen was rapidly passed through them. They were reduced with hydrogen once or twice a week, depending on the amount of gas used. The gas was passed through a strong solution of sodium hydroxide just previous to passing through the furnace.

In front of the thermostat a movable burette was arranged in such a manner that it could be moved to any electrode chamber or placed out of the way on one side of the thermostat. 5 mm. copper tubing which formed the connection to the nitrogen line was coiled to facilitate the easy moving of the burette without causing the copper tube to crack. No rubber tubing was used.

For the reduction of the indigo carmine an apparatus similar to that described by Clark and Cohen was used. The hydrogen was purified by passing through a Pyrex glass tube filled with copper which could be heated with a resistance coil. After reduction with platinum and hydrogen, the solution was transferred, without contact with air, to the movable burette. Reduced indigo solutions were prepared as follows: 520 mg. of synthetic indigo were added to a 500 cc. Pyrex distilling flask, to the side neck of which a Pyrex stop-cock was attached; 70 cc. of water and 30 cc. of normal sodium hydroxide were added to the flask with the indigo. Hydrogen was bubbled through the flask and out the side neck. To the side neck was attached a dropping funnel containing 100 cc. of water and 3 gm. of glucose. The hydrogen was permitted to bubble through the funnel in order to deoxygenate the solution. After the oxygen had been removed from the flask and the solutions, the alkaline solution containing the indigo was heated to boiling and the glucose solution was added in small portions. The indigo was rapidly reduced and the alkali was neutralized by the formation of hydroxy acids from the glucose. If the indigo is oxidized by bubbling air through 10 cc. of the solution and the precipitated indigo is filtered out, the solution is found to be almost neutral when phenolsulphonephthalein is the indicator. In the neck of the flask was placed a two-hole rubber stopper. Through one hole the hydrogen was admitted to the bottom of the flask and through the other a tube was passed which could be raised and lowered, and which communicated with the movable burette. In order to transfer the indigo to the movable burette, the glass tube connecting the flask with the burette was held stationary and the

rest of the apparatus was lifted up by a suitable frame built around the preheating glass tube containing copper and the distilling flask. The entire apparatus was raised to such a point that the delivery tube touched the bottom of the flask, and the hydrogen forced the solution through the delivery tube into the movable burette. The movable burette was then dehydrogenated by means of oxygen-free nitrogen, and the indigo solution was in a satisfactory condition to be added to the electrode chambers.

The preparation of 2-oxydihydroindole-3-propionic acid and its halogen derivatives has been described (6). The buffered solutions were prepared according to the phosphate table of Sörensen (2). In most cases a pH 7.4 was used by taking 80 cc. of M/15 disodium phosphate and 20 cc. of M/15 monopotassium phosphate. For the actual determination 100 cc. of the phosphate mixture were always used, and in cases in which the reduced forms of lactams were used, the dry sodium salt was placed in the electrode chamber and the phosphate solution was put into the movable burette. Oxygen-free nitrogen was then passed through the electrode chamber and the buffer solution for at least 45 minutes. The phosphate solution was then added to the dry powder in the electrode chamber.

The indigo carmine and the indigo were commercial preparations. The dibromoindophenol and 1-naphthol-2-sulfonate-indo-2,6-dichlorophenol were prepared in this laboratory by Dr. E. J. Witzemann, according to methods indicated by Clark, Cohen, and Gibbs (3, 4).

EXPERIMENTAL.

Unless otherwise indicated the following results were obtained in a solution buffered to a pH 7.4 which was secured by using 80 cc. of M/15 disodium phosphate and 20 cc. of M/15 monopotassium phosphate. The 100 cc. of buffer solution were deoxygenated in the movable burette by passing oxygen-free nitrogen through the solution for from 45 to 60 minutes. The material to be studied was placed in the bottom of the electrode chamber and the air in the electrode chamber was displaced by oxygen-free nitrogen. The deoxygenated buffer solution was then added to the electrode chamber, at no time coming in contact with atmospheric oxygen. As soon as the material was dissolved, observations were taken.

TABLE I.

Time.		Oxidation-reduction potentials.		
		Solution 1.	Solution 2.	Solution 3.
hrs.	*min.*			
	0	0.062	0.079	0.033
	9	0.069		
	21		0.080	
	23	0.067		
	26			0.036
	47	0.060		
	48		0.081	
1	2	0.054	0.081	
1	5			0.036
1	17		0.082	
1	21	0.054		
1	31		0.082	
1	35	0.060		
1	43		0.078	
1	51	0.062		
1	53			0.034
2	10	0.056		
2	20	0.086		
2	32			0.031
2	35	0.060		
2	48	0.061		
2	53			0.029
3	4		0.064	
3	10	0.059		
3	21		0.077	0.021
3	22	0.058		
3	37	0.053		
3	51	0.053		
3	58			0.022
4	15		0.080	
4	16	0.058		
4	35			0.025
4	36	0.054		
4	57			0.025
5	14	0.054		
5	23		0.077	
5	32	0.053		
6	25	0.048		
6	37		0.078	

In some cases heat was applied to the electrode chamber by directly heating with a Bunsen burner or an electric hot plate.

The dibromoindophenol was in each case an 0.2 per cent solution which is approximately 0.011 normal.

In order to show the drifting potentials and the difference between two solutions prepared in an apparently identical manner two samples each of 288 mg. = 0.0012 gm. equivalent of the monosodium salt of 4,6-diiodo-2-oxydihydroindole-3-propionic acid were added to two 100 cc. solutions of buffer of pH 7.4. The

TABLE II.

Time.		Oxidation-reduction potentials.		
		Solution 1.	Solution 2.	Solution 3.
hrs	*min.*			
	0	0.236	0.229	0.238
	11		0.230	
	17	0.235		
	20			0.235
	31		0.230	
	40			0.235
	46	0.235		
1	3			0.235
1	8	0.234		
1	14		0.232	
1	25			0.235
1	26	0.234		
1	45			0.235
1	49	0.234		
2	2		0.233	

readings taken at the times indicated show that widely differing potentials were produced in the two solutions (see Fig. 1).

An example of drifting potentials and data showing the inability to relate a difference in concentration and reducing potentials is given in Table I. In this a buffer of pH 7.8 was made by using 25 cc. of N/10 borax and 75 cc. of a mixture of boric acid M/5 and sodium chloride M/20. 85 mg. of 2-oxy-2,3-dihydroindole-3-propionic acid were added to Solution 1, 50 mg. to Solution 2, and 15 mg. to Solution 3. In addition the oxidized form of this lactam was added in the following amounts to the three electrode

chambers: to the first 15.2 mg., to the second, 50.5 mg., and the third 85.9 mg. The ratio of reduced to oxidized form in the first chamber was 15:85; in the second, 1:1, and in the third 85:15. The results show the extent of change in the reducing potential during 6½ hours.

In order to show the effect of concentration on the oxidation with dibromoindophenol 20.5 mg. = 0.002 gm. equivalent of 2-oxydihydroindole-3-propionic acid were placed in 100 cc. of buffer in the first electrode chamber. 123 mg. of this compound = 0.0012 gm. equivalent were placed in 100 cc. of buffer of pH 7.4 in the second chamber. A solution of 2,6-dibromoindophenol was added at the times indicated, reulting in the changes in the potential, as shown in Fig. 3. The results bring out the fact that dibromoindophenol oxidized the solutions in the first electrode chamber, producing less increase in the reducing potential with each addition of dibromoindophenol. The color of the solutions was in agreement with the electrode potentials.

The halogen derivatives of 2-oxydihydroindole-3-propionic acid in both their reduced and oxidized forms affect the electrode potential so little that the results obtained by the addition of these substances to the buffer produce no appreciable change in the potential from that of the buffer alone. Table II shows the results of three solutions. Solution 1 is the buffer alone, pH 7.4, Solution 2 is 48 mg. = 0.002 gm. equivalent of the monosodium salt of 4,6-diiodo-2-oxydihydroindole-3-propionic acid, and Solution 3 is the buffer and 360 mg. = 0.0002 gm. equivalent of the monosodium salt of 4,6-dibromo-2-oxydihydroindole-3-propionic acid in its oxidized form.

In order to bring out the stability of the oxidized form of the lactam 36 mg. = 0.0002 gm. equivalent of the 4,6-dibromo-2-oxydihydroindole-3-propionic acid were dissolved in 100 cc. of buffer containing 2 cc. of N/10 sodium hydroxide. At the times indicated a solution containing 4 mg. for each cc. of reduced indigo carmine was added. The addition of the first amount of indigo carmine was promptly followed by an increase in the reducing potential, and no poising action of the compound was shown. The second addition of indigo carmine raised the reducing potential to a slightly higher figure but did not cause the reduction of any of the lactam (see Fig. 4).

4,6-diiodo-2-oxydihydroindole-3-propionic acid is sensitive not only to dibromoindophenol, but is also affected by an increase in the temperature. The following four solutions were prepared by using the following amounts of the diiodo derivative in 100 cc. of phosphate buffer pH 7.4.

Solution 1. 46 mg. = 0.0002 gm. equivalent + 1 cc. N/10 sodium hydroxide.
Solution 2. 92 mg. = 0.0004 gm. equivalent + 2 cc. N/10 sodium hydroxide.
Solution 3. 184 mg. = 0.0008 gm. equivalent + 4 cc. N/10 sodium hydroxide.
Solution 4. 276 mg. = 0.0012 gm. equivalent + 6 cc. N/10 sodium hydroxide.

The solutions were all boiled, and after cooling the reducing potentials were found to be much higher than those given in Fig. 2 in

TABLE III.

Time.		Oxidation-reduction potentials.			
		Solution 1.	Solution 2.	Solution 3.	Solution 4.
hrs.	*min.*				
	0	0.135	0.099	−0.067	−0.086
	14	0.132	0.089	−0.078	−0.086
	34	0.129	0.079	−0.077	−0.086
1	9	0.125	0.068	−0.082	−0.086
1	20	0.120	0.056	−0.085	−0.084
1	41	0.118	0.050	−0.085	−0.079
1	58	0.116	0.044	−0.084	−0.077
2	25	0.114	0.037	−0.087	−0.071
2	58	0.108	0.027	−0.091	−0.067

which the solutions of this diiodo derivative were not boiled. Moreover, the solutions gave varying and drifting potentials after boiling which were much more marked than those in which no heat was used (Table III).

When hydrogen dioxide was added to a solution of 4,6-diiodo-2-oxydihydroindole-3-propionic acid, an unexpected result was obtained. The hydrogen dioxide caused a drop in the reducing potential and did not oxidize the lactam in a manner similar to the oxidation brought about by dibromoindophenol. Furthermore, it materially slowed down the oxidation of the lactam with di-

bromoindophenol subsequently added. The hydrogen dioxide solution was N/50. To each of the four electrode chambers 288 mg. = 0.0012 gm. equivalent of the diiodo lactam and 100 cc. of buffer pH 7.4 were added. At the times indicated 5 cc. of a solution of 0.2 per cent dibromoindophenol were added (see Fig. 5).

The 4,6-dibromo-2-oxydihydroindole-3-propionic acid reacts in a manner similar to the unhalogenated lactam with 2,6-dibromoindophenol. The results in Fig. 6 were obtained by using 232 mg. = 0.0012 gm. equivalent in 100 cc. of buffer of pH 7.4. The dibromoindophenol was added at the times indicated and produced the typical changes in the reducing potential.

6-Monoiodo-2-oxydihydroindole-3-propionic acid reacts with 2,6-dibromoindophenol in a manner entirely similar to the unhalogenated lactam. 200 mg. = 0.0012 gm. equivalent of 6-monoiodo-2-oxydihydroindole-3-propionic acid were dissolved in 100 cc. of buffer solution containing 6 cc. of N/10 sodium hydroxide. The dibromoindophenol added at the times indicated in Fig. 7 brought about the initial drop in the reducing potential followed by an increase to a point higher than that originally present.

Fig. 2 shows the reducing potential of the solution of the monosodium salt of 4,6-diiodo-2-oxydihydroindole-3-propionic acid. 288 mg. = 0.0012 gm. equivalent for each 100 cc. of buffer pH 7.4. The solution was allowed to stand 18 hours and 55 minutes with but slight increase in the reducing potential. The addition of dibromoindophenol at this time was followed by a slight decrease in the reducing potential and then by an increase. Further addition of dibromoindophenol produced similar effects.

SUMMARY.

1. The lactam 2-oxydihydroindole-3-propionic acid will easily lose two atoms of hydrogen and exist as 2,3-iminophenyl-α-glutaric acid. These two substances are the reduced and oxidized forms of this compound.

2. When a mixture of the reduced and oxidized forms is placed in a phosphate solution buffered to pH 7.4 it does not produce a potential which can be related to the concentration of the oxidized and reduced forms by the usual equation. The potentials are characterized only by fluctuations and drifts which cannot be

explained by changes in temperature, light, or the presence of impurities.

3. If mild oxidizing agents, such as some of the indophenols are added to the solution, a slow oxidation of the lactam takes place, resulting in an increase of the reducing potential to a point much higher than that given by the reduced form of the lactam alone. Subsequent additions of the oxidizing agent bring about a temporary decrease in the reducing potential which is followed by a return to the value which is given by the fully reduced form of the oxidizing agent added. The actual potential of the solution is determined at any time either by the ratio of reduced to oxidized indophenol, or if the dye is completely reduced, by the concentration of the reduced form of the dye.

4. By the use of 2,6-dibromoindophenol as an oxidizing agent the fluctuations and drifts in the potential are eliminated and solutions are produced which can be duplicated, giving results of quantitative value.

5. The addition of halogen atoms to 2-oxydihydroindole-3-propionic acid increases the speed of oxidation of the lactam but does not significantly change the potential of the solution after equilibrium has been reached.

6. At no time during the oxidation of the lactam is there any evidence of autocatalytic action. The oxidizing agent appears to react in stoichiometric proportion with the lactam. The speed of reaction, however, cannot be expressed by a mono- or dimolecular equation. Apparently an addition product is formed between the lactam and the oxidizing agent which is unstable, easily breaking down with the removal of two atoms of hydrogen from the lactam.

7. The increase in the reducing potentials of a solution after heating and the decrease after the addition of hydrogen dioxide suggest that this lactam is capable of existing in a highly reactive state, the changes in potential indicating alterations in the lactam brought about by the activity of the amine group when the pyrrolidone ring is open.

8. The addition of a small amount of hydrogen dioxide suppresses the speed of reaction of this lactam with dibromoindophenol, and hydrogen dioxide does not bring about oxidation of the lactam in a manner similar to the oxidation with dibromoindophenol.

9. Although 2-oxy-2,3-dihydroindole-3-propionic acid and α-(2,3-iminophenyl) glutaric acid are the fully reduced and oxidized forms of this indole-inone derivative, the mixture of the two compounds does not form a reversible oxidation-reduction system, and no substance has been found which will bring these two compounds into equilibrium with each other.

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INTERMEDIARY CARBOHYDRATE METABOLISM.

I. INFLUENCE OF INSULIN ON LEVULOSE AND GLUCOSE INTRAVENOUSLY ADMINISTERED.

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The problem of how insulin acts in the body offers an interesting theme for investigation, when one considers the action of insulin on two closely related sugars—the aldo sugar glucose and the keto sugar fructose (levulose).

It has been stated by Clark (1917): "When the pancreas of a dog is perfused aseptically with Locke's solution containing dextrose in physiological concentrations, the optical rotation of the perfusate is diminished but its reducing power is unaltered. . . . . These changes do not occur with levulose. . . . While the living heart can destroy both dextrose and levulose to some extent, the experimental results suggest that the enzyme or enzymes derived from the perfused pancreas have a specific action on dextrose and are responsible for certain essential steps by which dextrose is prepared for normal utilization." Mann and Magath (1921) later found a difference between levulose and glucose in experiments on hepatectomized dogs. "An animal which is practically moribund and about to die is restored to a seemingly perfectly normal condition within one minute after the injection of 0.5 gm. of glucose per kilo of body weight." But levulose had no restorative action whatsoever. After the isolation of pancreas enzyme as insulin Noble and Macleod (1923) tested in rabbits the curative action of different sugars on insulin shock and found that levulose "can *almost* be used as a substitute for glucose, the characteristic feature of its action . . . being that the convulsions readily relapse after an apparently prompt recovery following its injection. . . . . [But] these relapses may also occur some time after glucose. . . . . but in this case a further injection has almost invariably been found efficacious, which has not been found to be the case with levulose. With glucose also the recovery for some time following an injection is usually complete while with levulose the

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animal appears to be continually on the verge of convulsions." Herring, Irvine, and Macleod (1924) make a similar statement in their work on the action of different substances on insulin shock in mice.

Levulose not only behaves differently from glucose when it is added to a body containing an artificially increased quantity of insulin but if the two sugars are introduced into an organism with a natural normal content of insulin they behave differently. Bürger (1921) has introduced them intravenously in the same amount and has shown marked differences in the metabolic effect on the normal organism (dog). But even if these two sugars are introduced in the most natural way by the alimentary tract the heat production and respiratory quotients after their introduction behave in quite a different way as stated by all the workers who have tried to compare the metabolism of glucose and levulose introduced by mouth. The differences between the metabolism of these two sugars introduced by mouth are similar to those which develop when these sugars are introduced by vein in separate experiments.[1] This fact seems to be of importance in the theory of behavior of these two sugars in the body. The passage therefore through the wall of the alimentary tract seems not to play an essential rôle in the metabolism of these sugars either by causing a conversion of levulose into glucose, or a faster absorption of one of these sugars. Glycogen formation from both sugars is about the same and their respective glycogen-forming powers are not the cause of the difference. Also conversion of levulose into glucose does not play a rôle in this connection as has been shown most conclusively by Lusk (1915). It seems to be possible that in a *normal* organism glucose and levulose are taken as such into the gears of the metabolic mill and metabolized each in its own way.

Insulin alone has no power of converting levulose into glucose. Hepatectomized or insulinized dogs have plenty of insulin but respectively they either do not recover or recover less successfully when levulose is given. On the other hand the most severe diabetic converts levulose into glucose almost quantitatively although having little or no available insulin.

Our problem was to examine the action of insulin on the intermediary products of both sugars. What happens to glucose or to levulose when they are supplied to the cells intravenously at one and the same constant rate and at a rate similar to the rate at which they may be supplied to the cells from the alimentary tract following oral administration? Exclusion of the gastrointestinal tract simplifies the problem. What happens to these sugars when they are exposed to normal and abnormally increased quantities of insulin?

[1] The analogy is striking when Bürger's Experiments 5 and 7 are compared with Dr. Boothby's and the author's experiments on glucose and levulose metabolism which are about to be published.

Method.

Nine healthy female dogs were used. They were fed a constant ration of meat and bread. They were fasted for about 20 hours before the experiment. Into these dogs sugar solutions were injected by vein at a constant rate over long periods of time by means of the volumetric pump developed by Woodyatt (1917, 1920). Throughout the entire study the injection rate was 2 gm. per kilo of body weight per hour.

The glucose used was glucose C.P., Corn Products Refining Company, which was considered satisfactory without any purification. The levulose used was levulose (technical 90 per cent), Pfanstiehl, white powder, perfectly soluble in water, which was repurified in the laboratory by repeated shaking with charcoal which removed most of the yellow-brown color occurring when directly dissolved. Both sugar solutions were sterilized by passing through a Berkefeld filter. Both glucose and levulose solutions checked within 1 per cent by reduction and polarimetry, which assured a minimum of impurities present. Glucose solutions were water-clear when in 20 per cent solution, levulose solutions had a slight yellow color in the same dilution. Any heating of the sugars was carefully avoided. Sugar solutions were always freshly prepared using for solutions recently prepared double distilled water. Saphenous veins were used for injection. Dogs were always trained by preliminary experiments, so that they lay quietly over long periods of time on the board.

Sugars were determined in the urine in the beginning by reduction and polarimetry, which in the case of glucose agreed perfectly. Even when the glucose excretion fluctuated, the specific rotation of the glucose excreted remained constant as shown by constant agreement of polarimetry and reduction (within 2 per cent) in all the samples, corresponding for glucose with $+52.5°$ specific rotation. The same constancy of specific rotation of excreted glucose was observed after insulin injection. When after insulin injection glucose excretion in the urine was very small the sugar excreted was determined by reduction.

In the case of levulose, reduction gave slightly larger values than polarimetry. The latter detected 90.3 to 92.4 per cent of the amount of sugar found by reduction and this proportion

remained fairly constant after insulin injection (Table I) showing that the sugar excreted was always in the same state. It did not correspond to our conventional figure for the specific rotation of levulose, − 92–93°, because of the probable admixture of other reducing substances (Folin and Berglund, 1922) which had a different specific rotation than pure levulose in equilibrium and were not glucose because insulin had no influence on them.

TABLE I.

Agreement between Levorotation and Reduction when Insulin Was Injected during Continuous Intravenous Injection of Levulose at a Rate of 2 Gm. per Kilo per Hour. Dog 1.

Time.	Levulose excreted in urine per hr.		Reduction recovered by polarimetry.
	By reduction.	By polarimetry.	
hrs.	*gm.*	*gm.*	*per cent*
2	1.59	1.44	90.1
3	1.49	1.38	92.5
4	1.34	1.23	91.6
20 units of insulin injected.			
5	1.40	1.24	89.2
6	1.30	1.15	88.0
7	1.40	1.25	89.5
8	1.31	1.18	89.9
9	1.31	1.17	88.8
40 units of insulin injected.			
10	1.44	1.31	91.1
11	1.46	1.34	91.9
Average.....			90.3

Since it was found that when glucose was excreted, polarimetry gave fairly satisfactory results and with levulose a discrepancy of less than 10 per cent which was negligible for our purposes, polarimetry was used exclusively with the consideration in mind that, as the urines of those dogs were normal and not appreciably optically active (rotation was determined in the urine of every dog before the experiment) and as the specific rotation of the sugars excreted remained constant, polarimetry could be con-

sidered a fairly reliable and time-saving method for this particular study. Bertrand's method was used as a standard reduction method and a Schmidt-Haensch polariscope with precision of 0.01°.

For drawing blood samples superficial veins on the opposite leg were used, which were exposed in such a way as to be accessible without the least change in the position of the dog. Blood sugar was determined by the Folin-Wu method. In such conditions experiments could be carried on over a period of 16 hours. Levulose when injected over long periods of time influenced the circulation in some dogs unfavorably, producing some dyspnea and cyanosis. This did not appear however during the important parts of the experiments here reported. Therefore the toxic action of levulose or rather of levulose impurities does not need to be taken into consideration in the following work.

In levulose experiments the urine sugar was tested both by levorotation and by Seliwanoff's reaction which was always strongly positive when definite levorotation appeared in the urine.

Results.

Of the number of experiments performed (eighteen) only the most satisfactory from the technical standpoint are here given. When glucose is administered to a dog by a pump for continuous intravenous injection at a rate of 2 gm. per kilo body weight per hour some of this glucose appears in the urine, the rest is metabolized in the tissues. Usually during the first 1 to 3 hours of injection excretion is yet stabilized. Afterwards when conditions come to an equilibrium excretion becomes virtually constant. (Preliminary periods before the insulin injection have been made long enough to insure constant excretion.) At this point insulin was injected in an amount which was supposed to give maximal effects.

Chart 1 shows two experiments performed on the same dog, No. 3, weighing 11.7 kilos and receiving therefore $11.7 \times 2 = 23.4$ gm. of glucose intravenously per hour (Experiment 4). The amounts of glucose eliminated in the urine during early hours of injection (2nd to 5th hours) were 3.20 gm. of glucose per hour as average. This amount of glucose eliminated in the urine

constitutes 13.7 per cent of the amount of sugar injected per hour. At the end of the 5th hour 100 units of insulin were injected; glucose excretion during the next hour gave the same figure as the average of preceding hours, namely 3.23 gm., while during subsequent hours the curve of sugar excretion drops down as a

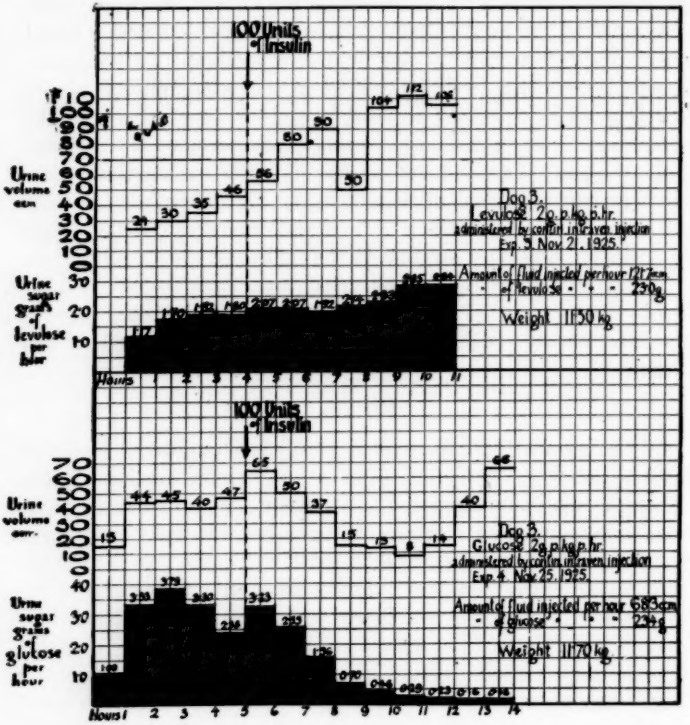
100 Units of Insulin
Urine volume cm.
Urine sugar grams of levulose per hour
Dog 3.
Levulose 2g. p.kg.p.hr.
administered by contin. intraven. injection
Exp. 3. Nov. 21. 1925.
Amount of fluid injected per hour 121.7 cm. of levulose + ½ = 230g
Weight 11.50 kg.
Hours
100 Units of Insulin
Urine volume cm.
Urine sugar grams of glucose per hour
Dog 3.
Glucose 2g. p.kg.p.hr.
administered by contin. intraven. injection
Exp. 4. Nov. 25. 1925.
Amount of fluid injected per hour 68.5 cm. of glucose + ½ = 234g
Weight 11.70 kg.
Hours

CHART 1.

curve of compound interest, which plotted on semilogarithmic paper with hours placed equidistant on the ordinate, gives in most cases a straight line.

The urine volume, which during 4 hours preceding the insulin injection averaged 44 cc. and was fairly constant, representing 64 per cent of the fluid injected, dropped in close analogy to the

glucose excretion to as little as 8 cc. during the 6th hour after insulin injection and increased during the 13th and 14th hours of experiment. There was a water retention going hand in hand with the decrease of urine sugar elimination.

In the same dog levulose was injected at the same rate but dissolved in larger amounts of water (Dog 3, Experiment 3, Chart 1). As with glucose some levulose escapes in the urine. This amount came to constancy before insulin injection; average excretion from 2 to 4 hours was 1.77 gm. of levulose per hour. This amount of levulose in the urine was 7.7 per cent of that injected in the same time. In this case levulose was better retained than glucose. Then 100 units of insulin were injected and contrary to the glucose experiment the amount of levulose excreted in the urine remained constant showing no decrease whatsoever, whereas with glucose injection insulin almost abolished excretion of glucose.

Evidently insulin was able to depress the sugar excretion in the urine to the trifling amount of 0.16 gm. per hour which was 0.7 per cent of the amount of glucose administered per hour and constituted an amount which could not be removed by any amount of insulin added to the body. But on the contrary insulin was unable to influence to the slightest extent the levulose excretion in the urine even when administered in such an excessive dose as 8.5 units per kilo of body weight. No visible signs of insulin reaction were observed in this dog.

Urine volumes during this levulose experiment show a constant progressive tendency which could be ascribed to the large amount of water which was introduced into the body with the levulose (much larger than in corresponding glucose experiment). Some of this water was retained in the body and when retention increased, more and more urine was eliminated. This fact perhaps could account for a certain rising tendency of the levulose excretion towards the end of the experiment if levulose had been retained before that time as it may well have been.

Chart 2 shows two experiments conducted on different dogs. In Dog 4 (Experiment 2) glucose excretion came to constancy during the 2nd to 5th hours of the experiment, the excreted amount being 2.27 gm. as average per hour, this being 10.6 per cent of the hourly amount administered intravenously in this dog

(22.0 gm.). After insulin was injected the sugar in the urine began to drop, showing the same shape of curve as in the preceding experiment. Insulin action did not come to its peak before the experiment was stopped (an accident).

In the same dog a short experiment was made (Dog 4, Experiment 3, not charted) with levulose injection without insulin. The excretion of levulose in the urine obtained was 2.00 gm. as average from 3rd to 6th hours, this being 8.8 per cent of the amount of levulose administered intravenously in the same time.

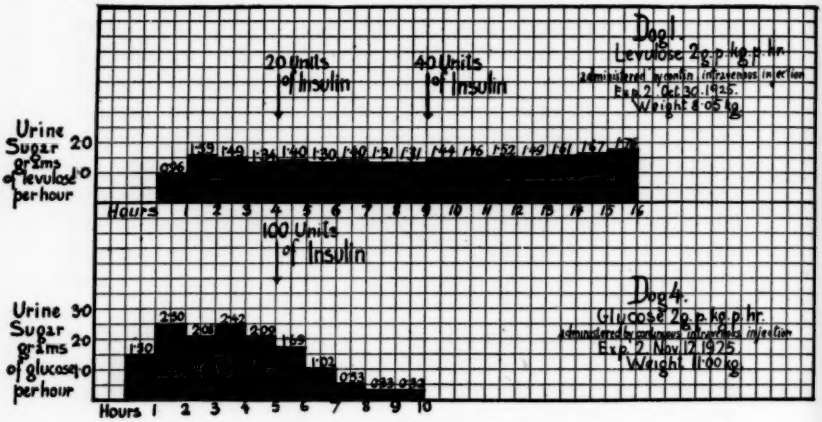
Dog 1.
Levulose 2g. p. kg. p. hr.
administered by continuous intravenous injection
Exp. 2. Oct. 30. 1925.
Weight 8.05 kg.
20 Units of Insulin
40 Units of Insulin
Urine Sugar grams of levulose per hour
0.26 1.39 1.49 1.34 1.40 1.30 1.40 1.31 1.31 1.44 1.46 1.52 1.49 1.61 1.57 1.78
Hours 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
100 Units of Insulin
Dog 4.
Glucose 2g. p. kg. p. hr.
administered by continuous intravenous injection
Exp. 2. Nov. 17. 1925.
Weight 11.00 kg.
Urine Sugar grams of glucose per hour
1.30 2.50 2.03 2.62 2.08 1.58 1.02 0.53 0.33 0.32
Hours 1 2 3 4 5 6 7 8 9 10

CHART 2.

In the case of this dog the yields of levulose and glucose in the urine were about the same.

The other experiment in Chart 2, made on on Dog 1 (Experiment 2) is interesting because levulose was administered intravenously over a period of 16 hours, during which time 258 gm. of levulose were injected in a small pregnant female weighing 8.05 kilos. During the 2nd to 4th hours of injection levulose excretion came to constancy, giving 1.47 gm. of levulose as average per hour. The dog received 16.1 gm. of levulose per hour, therefore the 1.47 gm. in the urine was 9.1 per cent of the amount injected. Then 20 units of insulin were injected subcutaneously. Subsequent figures show during 5 to 9 hours 1.34 gm. as average

per hour. Again 40 units of insulin were injected and the levulose excreted during 10th to 16th hours was 1.57 gm. as average per hour.[2]

After the experiment was over the dog excreted during the next few hours an amount of 0.39 gm. of levulose which was less than 2 per cent of the total amount excreted during the experiment itself and 0.15 per cent of the amount of levulose injected during 16 hours. This after excretion of levulose was therefore neglected in other experiments.

One reflection on the metabolism of this dog may be of interest here. It is startling to note the large amounts of levulose that can be utilized by that ever working machine—the cells of the body. After a continuous supply which was 3.8 times larger than the basal metabolic requirement of this dog (after subtraction of the amount excreted in the urine), lasting 16 hours, the cells were not tired. The amount of levulose supplied here recalculated on the basis of basal metabolic requirement would mean for a normal man of 75 kilos body weight a supply of 73 gm. of levulose per hour or 1168 gm. of levulose during 16 hours, equivalent to 4438 calories. Even a hard working lumberman could get his daily need of calories by vein in the form of pure levulose. Levulose is therefore an excellent foodstuff.

Charts 3 and 4 represent a set of experiments on the same dog, No. 6, in which besides the amount of sugar injected also the volume of fluid was adjusted identically in the glucose and levulose experiments. In addition to the usual measurements of the amount of sugar in the urine and urine volume, blood samples were drawn in order to eliminate the possibility that the decrease of sugar in the urine in glucose cases was not due to retention and to elucidate what happens to the blood sugar in levulose cases.

The first experiment (Chart 3, Dog 6, Experiment 1) was performed as control without drawing blood samples in order to show that, whether blood is drawn or not, the sugar excretion remains the same. In this experiment the glucose excretion did not come to constancy but showed a continual increase, which could not register the insulin effect because this acts in a contrary manner. Nevertheless, we are justified in taking the aver-

[2] Dog behaved perfectly quietly during the whole experiment; gave birth next morning to two dead puppies.

age of the 4th to 6th hours, which gives an approximate idea how in this dog, glucose was utilized; the amounts for the time indicated being 3.16 gm. per hour, which is 15.8 per cent of the glucose supply per hour.

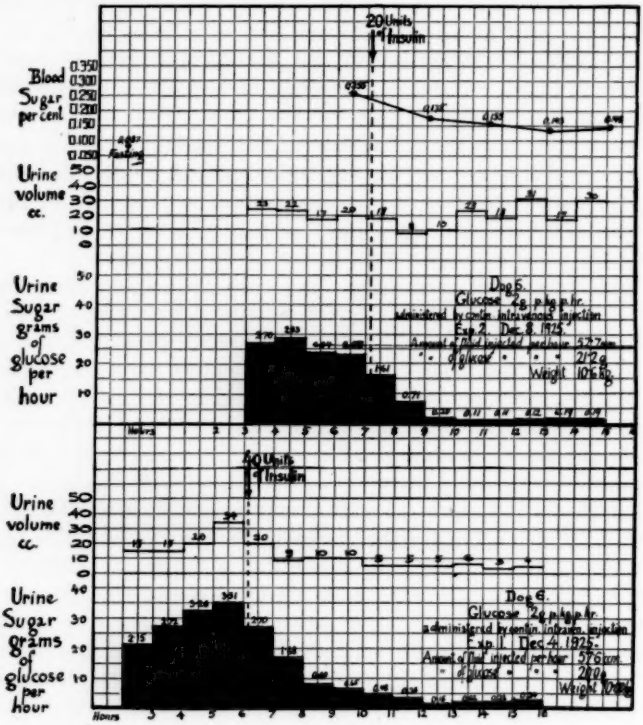
20 Units of Insulin
Blood Sugar per cent
Urine volume cc.
Urine Sugar grams of glucose per hour
Dog 6.
Glucose 4 g. p. kg. p. hr.
administered by contin. intravenous injection
Exp. 2. Dec. 8. 1925.
Weight 10½ kg.
40 Units of Insulin
Dog 6.
Glucose 2 g. p. kg. p. hr.
administered by contin. intravenous injection
Exp. 1. Dec. 4. 1925.
Hours

CHART 3.

After injection of 40 units of insulin again the glucose excretion in the urine slowly vanished at a rate comparable to that in preceding experiments until an insulin-resistant minimal amount of glucose, 0.16 gm. in the urine, was obtained which amounted to 0.8 per cent of the amount injected per hour in the same time and was only 5.1 per cent of the average amount of

glucose excreted before insulin injection. After the lowest point has been reached, the glucose in the urine began again to show a slow tendency to increase. The urine volume dropped from an average of 23 cc. during the 4th to the 6th hours to 5 cc. which persisted until the end of the experiment.

In the second experiment (Chart 3, Dog 6, Experiment 2) during glucose injection blood sugar determinations were made. Glucose excretion before insulin injection gave 2.53 gm. as an

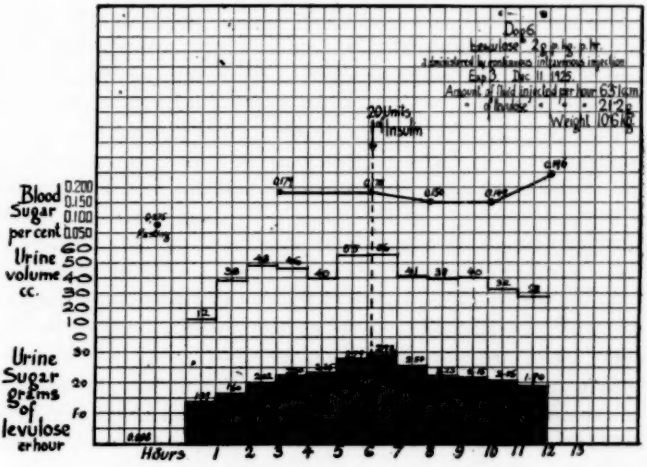

CHART 4.

average (4th to 7th hours). The corresponding blood sugar was 0.255 per cent. This amount of glucose in the urine was 11.9 per cent of the amount supplied by vein in the same period.

After injection of 20 units of insulin it became evident that insulin does not cause retention of glucose in the blood. On the contrary, with the drop in the urine glucose curve, an identical curve appears for the blood sugar, which in this case consists largely of glucose.

The blood sugar curve apparently reflects a drop in the urine sugar curve. The total drop of blood sugar during insulin action was from 0.255 to 0.143 per cent, which is 0.112 mg. The urine sugar did not entirely disappear (0.11 gm. of glucose per hour),

coincidently with the persistence of the blood sugar above the normal fasting level, which was in this dog 0.087 per cent. The urine volume in this case showed only a transitory decrease after insulin injection; namely, from 20 cc. to 8 cc.

In the levulose experiment (Chart 4, Dog 6, Experiment 3) the whole mechanism of levulose behavior towards insulin is clearly shown. Levulose excretion before insulin injection shows as in the glucose experiment in this dog (Chart 3, Experiment 1) a certain rising tendency.

During the 3 hours preceding insulin injection 2.46 gm. of levulose were excreted hourly, amounting to 11.6 per cent of the hourly intake and corresponding to the results of the preceding glucose experiment. After injection of 20 units of insulin, about 2 units per kilo, the levulose excretion did not show an important drop. The slight tendency in this direction towards the end of the experiment was accidental and due to slight retention of levulose, as shown by the final rise in blood sugar and decrease in urine volume, and cannot be attributed to insulin action because in other experiments there is a rise in levulose excretion.

As insulin seems not to affect the curve of levulose excretion in the urine, it might be expected that it would not affect the blood sugar when levulose constitutes the chief part. But it is evidently not so. Even if the blood is flooded with levulose this ketose does not replace completely the normal blood sugar composed in chief part of glucose and representing in this experiment 0.075 per cent (the fasting blood sugar concentration observed immediately before this experiment). This glucose circulates with levulose and is evidently metabolized selectively by insulin to as great an extent as in the fasting dog. In this experiment the selective action of insulin on the glucose fraction of the blood sugar without corresponding action on urine sugar may be observed.

Hour.	Blood sugar.	Levulose in the urine.
	per cent	*gm.*
3	0.179	2.24
	20 units of insulin.	
8	0.150	2.23
10	0.149	2.06

The urine volume shows constancy and is identical both after and before insulin injection. Towards the end of the experiment the urine volume shows a decrease. The end of this experiment gives in miniature a picture of sugar retention; the urine sugar

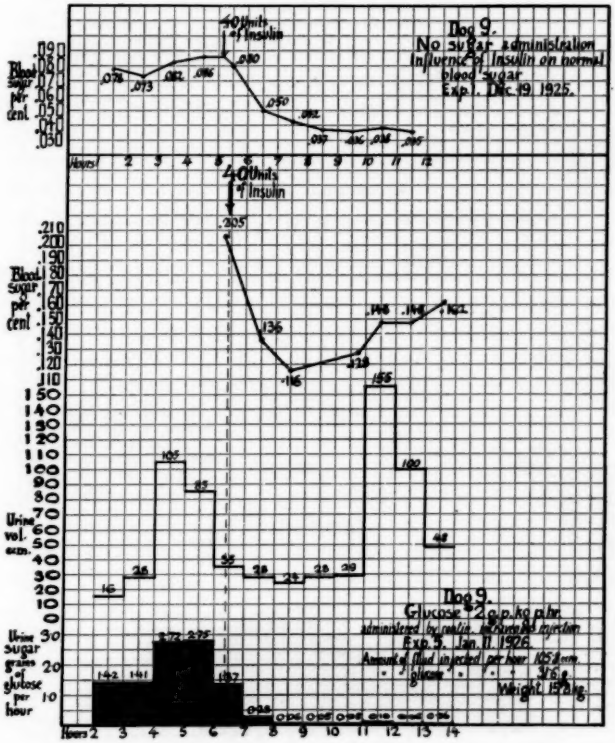
Dog 9.
No sugar administration
Influence of insulin on normal blood sugar
Exp. Dec 19 1925.
40 Units of Insulin
Hours
Blood Sugar in per cent
Urine vol. ccm.
Urine sugar grams of glucose per hour
Dog 9.
Glucose 2 g.p.kg.p.hr.
administered by subcut. intermittent injection
Exp. 5. Jan. 11. 1926.
Amount of fluid injected per hour 105.8 ccm.
Weight 15.2 kg.

CHART 5.

decreases with the urine volume, but at the same time the blood sugar increases.

In the glucose and levulose experiment (Dog 6, Experiments 2 and 3) identical amounts of sugar were injected amounting to 21.2 gm. but the blood sugar during glucose injection was 0.255 per cent and when levulose was injected 0.179 per cent, both be-

fore insulin injection, a difference of 76 mg. The volume of fluid injected was fairly close in both instances, with glucose 57.7 cc. and with levulose 63.1 cc. Nevertheless, the urine volume with levulose was twice that of glucose.

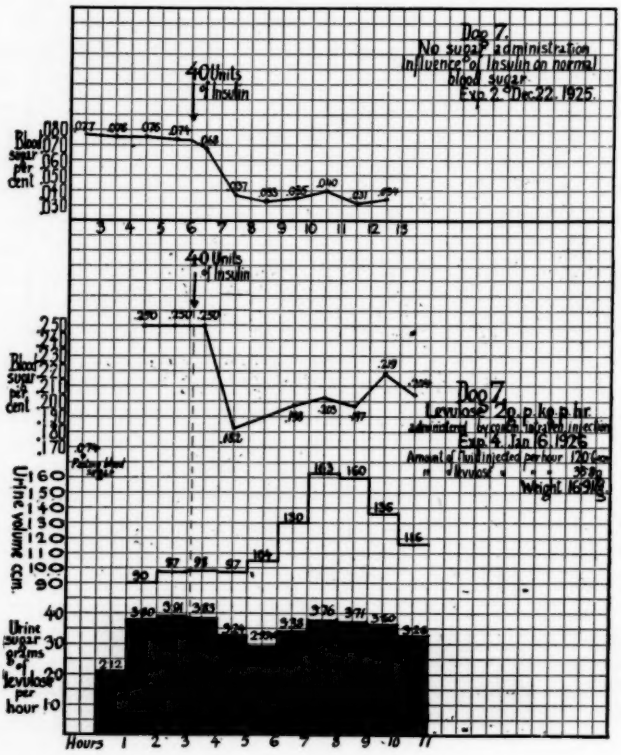
Dog 7.
No sugar administration
Influence of Insulin on normal blood sugar
Exp. 2. Dec. 22. 1925.
40 Units of Insulin
Blood sugar per cent
Dog 7.
Levulose 2g. p. ke. p. hr.
Exp. 4. Jan 16. 1926
Urine volume cm.
Urine sugar grms of levulose per hour
Hours

CHART 6. * Accidental slowing of the injection rate corrected during the next hour.

In Chart 3, in glucose experiments the trend of the curve in Experiment 1 is increasing, in Experiment 2 decreasing. Insulin however caused in both cases an immediate drop during the

1st hour after injection (period of subsequent decrease being longer in the case where insulin action met a higher sugar excretion (Experiment 1)), showing how quickly insulin acts on glucose metabolism.

Charts 5 and 6 will throw additional light on the manner in which insulin acts during glucose and levulose injection.

In Chart 5 (Dog 9, Experiment 5) glucose was injected at the usual rate and figures for sugar excretion in the urine were, during the 5th and 6th hours, 2.74 gm. average per hour, 8.7 per cent of the amount injected. After insulin injection it dropped in the usual manner. The blood sugar shows a depression of similar magnitude to that in the preceding experiments, dropping during the 2nd hour about 89 mg. per 100 cc. and during the 4th hour 77 mg. In the following hours the blood sugar increased, causing no corresponding increase in sugar excretion in the urine. The urine volume shows a marked decrease during the hours following insulin injection. In order to observe the relationship between the drop of the blood sugar curve during glucose injection and without injection of sugar, the same dose of insulin was injected in the same animal during fasting. The curve is shown in Chart 5 (Dog 9, Experiment 1). The blood sugar dropped from 0.086 per cent to about 0.037 per cent from the 4th hour on after insulin injection, a drop of 49 mg. It is clear that the absolute magnitude of the drop was larger during glucose injection.

Chart 6 represents an experiment with levulose injection (Dog 7, Experiment 4). Levulose excretion became constant during the 2nd and 3rd hours amounting to 3.88 gm. average per hour, this being 11.5 per cent of the supply. After insulin injection the levulose excretion remained constant (except during the 3rd hour after insulin injection when the injection rate slowed up accidentally). The urine volume immediately following the insulin injection showed no change and later began to increase. The blood sugar dropped from 0.250 to 0.182 per cent during the 2nd hour after insulin injection and became stable during the 4th to the 6th hours after injection, showing an absolute decrease of about 50 mg. per 100 cc. The experiment made on the same animal with the same amount of insulin without sugar administration showed a drop in blood sugar during the 3rd to the 7th hours amounting to 40 mg. per 100 cc. (Chart 6, Dog 7, Experiment 2).

It seems reasonable to assume that these drops in the blood sugar, one with levulose injection and the other without it, are of similar magnitude.

Striking from the standpoint of the relationship of the blood sugar level to the urine sugar excretion rate is a comparison of the two hourly periods before insulin and after insulin and their corresponding blood sugars. Almost identical amounts of levulose appear in the urine in the two periods, whereas the blood sugar level differs (Table II).

In such a way metabolism of these two sugars seems to be in a certain sense independent of each other, one of them can be stimulated without stimulation or inhibition of the other one. Cells metabolizing levulose at a certain rate can take up more burden by metabolizing glucose in addition, without appreciably

TABLE II.

Hour.	Levulose in urine.	Blood sugar.
	gm.	*per cent*
2	3.80	0.250
3	3.91	0.250
	40 units of insulin.	
8	3.76	0.203
9	3.71	0.197

changing the rate of levulose utilization. Levulose excretion does not increase after insulin injection.

Of course it is difficult in long experiments with continuous intravenous injection to avoid some fluctuation of the amount of sugar excreted. These fluctuations are frequently due as Woodyatt[3] has shown to temporary retention of unchanged sugar in the body in fluids not in active circulation, or to the flushing out of sugar so retained during previous periods. They may also be due in part to changes in the rate of utilization.

DISCUSSION.

The rate chosen for injection approached the rate at which these sugars can be supplied to the tissues from the alimentary tract when they are given by mouth in sufficient amounts. There-

[3] Woodyatt, R. T., personal communication.

fore, from this standpoint the amount of sugar supplied to the tissues was approximately physiological (Sansum and Woodyatt, 1917). In normal conditions a fairly constant amount of both sugars was eliminated in the urine (Table III).

A little more glucose was eliminated than levulose in this series of experiments, but practically the amounts eliminated approximate 10 per cent in both cases. This means that in the normal metabolism with its physiological insulin content both sugars when supplied directly to the cells at equal rate are taken up at about the same rate in metabolism, since the difference between the rate of injection and the rate of excretion in these experiments represents the rate of utilization, provided the excretion rates

TABLE III.

Dog No.	Experiment No.	Amount of sugar eliminated in urine.	
		Glucose.	Levulose.
		per cent	*per cent*
1	2		9.1
3	4 and 3	13.7	7.7
4	2 " 3	10.6	8.8
6	2 " 3	11.9	11.6
6	1	15.8	
7	4		11.5
9	5	8.7	
Average		12.1	9.7

were virtually constant. This seems to be an important point in the consideration of the metabolism of both sugars beyond the gastrointestinal tract. Blumenthal (1905) who injected both sugars intravenously in short intervals of time (every 15 minutes) found also that the tolerance of the tissues for glucose and levulose was about the same. Both sugars in normal conditions are fixed at similar rates by the tissues but their final fate in the cells is different in both cases, as shown by different respiratory data (Bürger, 1921) and the behavior of both sugars when the insulin content of the body is artificially increased during the intravenous administration of both sugars.

When the insulin content of the body is artificially increased

the two sugars show completely different behaviors. In the case of glucose the stream of this sugar to the kidneys is, in effect, shifted towards the tissues, leaving only a trifling amount of glucose in the urine. This process occurs in a regular way, suggesting enzyme action.

The tissues, however, are not able to metabolize all the glucose injected at this rate whatever the amount of insulin administered. A certain amount of glucose always escapes in the urine. This is probably due to the fact that during intravenous glucose injection into the saphenous vein the glucose entering the vein

TABLE IV.

*Amount of Glucose Which Escapes in Urine at the Peak of Insulin Action during Continuous Intravenous Injection of Glucose.**

Dog No.	Experiment No.	Hr. after insulin injection.	Amount of glucose injected.	Amount of glucose in urine.	Amount escaped as per cent of amount injected.	Utilization of sugar improved after insulin injection:		Amount of insulin injected per kilo of body weight in units.
						From.	To.	
			gm.	*gm.*		*per cent*	*per cent*	
3	4	8	23.4	0.16	0.7	86.3	99.3	8.5
6	1	7	20.0	0.16	0.8	84.2	99.2	4.0
6	2	4	21.2	0.11	0.5	88.1	99.5	1.9
9	5	4	31.6	0.05	0.2	91.3	99.8	2.5
Average.....					0.55	87.5	99.45	

* Glucose experiment on Dog 4 is not included here because in this experiment insulin action did not come to its peak before the experiment had to be stopped.

passes via the inferior cava to the right heart, through the pulmonary circuit into the left heart, and thence into the aorta, with little or no depletion, so that an intrasaphenous injection of sugar is in effect an intraaortic injection. Some of the aortic blood rich in the injected sugar passes directly via the renal artery to the kidneys before it has been in any other tissue where it could be acted upon by insulin.

As injected insulin may increase the utilization of glucose in a normal animal from 87.5 to 99.45 per cent (under the conditions described) implying almost complete utilization of the large amount of sugar injected (Table IV), this shows that the amount

of insulin which a normal organism is able to produce is not the maximal we could use. Insulin injection discloses latent metabolic ability of the cells to utilize glucose in excess of anything that occurs in ordinary life.

Different doses of insulin ranging from 1.9 to 8.5 units per kilo of body weight had similar effects, from which it can be concluded that certainly in these experiments the maximal utilizable dose of insulin (per unit of time) was injected. In Table IV can also be noticed a certain influence of the level of sugar excretion, before insulin injection, on the final insulin effect. If the amount of glucose excreted with regard to the amount of glucose injected was smaller it was easier for insulin added to the body to depress the glucose excretion in the urine.

On the contrary, levulose in the presence of artificially increased insulin behaved indifferently. It might be objected that insulin, therefore does not affect the levulose excretion in the urine because the rate of conversion of levulose into glucose in the cells of the body is slower than the rate of disposal of glucose by insulin, and levulose as "body foreign" material is excreted in the urine in the same amount after insulin injection as before. But if levulose could be converted into glucose in the cells of the body and undergo chemical changes similar to those of glucose, the respiratory and energy exchange of both sugars should be similar. As has been stated in the introductory remarks, this is not true.

It might be a logical speculation from the present experiments that, in the normal organism, little if any levulose goes over directly into glucose because if it did, to any appreciable extent, insulin injected would help to metabolize the glucose coming from the levulose and in this way remove glucose from the equilibrium (if it is a simple equilibrium) between levulose and glucose. One of the end-products (glucose) being removed, reaction would be shifted towards glucose, more levulose would go over into glucose and be metabolized as glucose by insulin with the result that levulose excretion in the urine would be markedly decreased. Nothing like this happens.

Isaac (1914) found that in the surviving liver of a normal dog levulose may be almost completely converted into glucose. This fact shows that isolated organs of a normal dog have the power of converting levulose into glucose to a large extent. But whether,

and if so to what extent, they exercise it in a normal living animal is another matter.

Dr. Woodyatt suggested that the statement about independence of levulose from insulin action would be stronger if supported by experiment to show that levulose can be utilized by the body in the absence of insulin. If insulin is not directly or indirectly necessary for levulose utilization, or if the latter is not in any sense dependent on insulin, it should be possible to demonstrate the utilization of large amounts of levulose in the absence of insulin; *i.e.*, in cases of diabetes of the most severe type.

Theoretically levulose metabolism is correlated and depends on the metabolism of all the other substances in the body and above all on glucose and indirectly on insulin. The relationship to glucose is either direct by conversion of fructose into glucose, as occurs in an almost quantitative way in most severe types of human and experimental diabetes, and perhaps to a small extent in a normal individual, or indirect through glycogen phase, as it is chiefly in a normal and perhaps to a small extent in a severely diabetic animal. The author feels, however, that strictly from the standpoint and under conditions of the facts presented above, there is no evidence of influence of insulin on levulose metabolism, whereas at the same time there is a remarkable influence on the glucose metabolism, all the other conditions being equal.

Thus insulin presents itself to us as a new means for the investigation of the intermediary carbohydrate metabolism because of its predilection for one precisely determined substance.

The author wishes to express to Dr. R. T. Woodyatt his great indebtedness for suggestions, criticism, and all the facilities for the accomplishment of this work.

SUMMARY.

The following conclusions apply only when glucose and levulose are injected into normal dogs continuously at the rate of 2 gm. per kilo of body weight per hour.

1. When glucose and levulose are introduced intravenously in the presence of a normal content of insulin in the body, a certain amount of these sugars (about 90 per cent in this group of experi-

ments) is chemically changed in the tissues, the rest, amounting to about 10 per cent (9.7 per cent as average for levulose, 12.1 per cent as average for glucose), escapes in the urine.

2. When glucose is injected, glucose appears in the urine; when levulose is injected, levulose is the sugar appearing in the urine. There is no evidence of any important amount of glucose in the urine when levulose is injected, and *vice versa*.

3. When these sugars are injected in the same animal in separate experiments at the same rate, the blood sugar content is much higher with glucose than with levulose.

4. There is no deposition of levulose in the body like a "body foreign" material under the conditions described during periods in which the rate of excretion is virtually constant. After injection of levulose is stopped, there are traces of levulose eliminated in the urine during a few hours amounting to 0.15 per cent of the total amount of sugar injected.

5. When the insulin content of the body is artificially increased during glucose injection, glucose excretion in the urine decreases in a curve resembling an enzyme action curve from 10 per cent of the supply of glucose to less than 1 per cent of the supply. The greatest insulin effect appears in different intervals of time varying from 4 to 8 hours after injection, and does not depend upon the dosage of insulin between limits of 1.9 to 8.5 units of insulin per kilo of body weight. The drop in sugar excretion appears in most cases during the 1st hour after the injection of insulin.

6. Insulin injected in any amount causes no change whatsoever in the levulose excretion during levulose injection.

7. Insulin injected causes a marked drop in the blood sugar curve when glucose is injected and a smaller drop when levulose is injected; this last being attributable to the drop of glucose preexisting in the blood before levulose injection.

8. The specific rotations of glucose and levulose excreted in the urine remain constant with and without insulin injection.

9. Even a sugar so closely related to glucose as levulose does not act in the normal organism as does glucose, but in its own way independent of insulin (under conditions described).

10. Insulin presents a new means for the investigation of the intermediary carbohydrate metabolism, being a hormone specifically set up for glucose.

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COLLOIDAL PROPERTIES OF THE SURFACE OF THE LIVING CELL.

I. CONDUCTIVITY OF BLOOD TO DIRECT ELECTRIC CURRENTS.

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(Received for publication, April 6, 1926.)

Stewart showed that blood cells act as insulators, but his and most later measurements were made with alternating currents, and since the mathematics is more complicated than with direct currents, the results with alternating currents are reserved for the second paper of this series.

Clerk Maxwell[1] devised a formula for conductivity of a uniform suspension of spheres of equal radii in a medium

$$R = R_2 \times \frac{2R_1 + R_2 + \rho(R_1 - R_2)}{2R_1 + R_2 - 2\rho(R_1 - R_2)}$$

in which R is the specific resistance (resistance of a cube of 1 cm. edge) of the suspension, R_1 of the spheres, R_2 of the medium, and ρ the ratio of the total volume of the spheres to that of the suspension (volume of spheres per cc.). Clerk Maxwell pointed out that the suspension must be dilute (radii of spheres small compared to their distances from one another) in order for the formula to hold. McClendon[2] attempted to test, experimentally, a formula derived from a paper by Breit.[3] MacDougall[4] used the same formula as Maxwell as indicated by McClendon.[5] It now appeared that Maxwell's, MacDougall's, and Breit's forms of

[1] Maxwell, J. C., Electricity and magnetism, Oxford, 3rd edition, 1892, i, 440, equation 17.

[2] McClendon, J. F., *J. Biol. Chem.*, 1924, lix, p. lvi.

[3] Breit, G., *Konin. Akad. Wetensch., Amsterdam*, 1922, xxv, 293.

[4] MacDougall, F. H., *Science*, 1924, lix, 403.

[5] McClendon, J. F., *Science*, 1924, lx, 523.

mathematics were essentially the same. The fact that they did not check with my measurements on blood might be explained by the fact that the corpuscles are not spheres, but since Clerk Maxwell states that the formula holds only for dilute suspensions it was thought best to use a graphical method for comparing the results rather than making an analytical study of them. Fricke[6] published a formula and I sent a copy to Dr. Breit who kindly examined it for me and replied: "Fricke's formula does not check with MacDougall's or mine. His error is made essentially in equation (4). This equation presupposes that the effective conductivity is the average conductivity (a volume average) which has no logical foundation. MacDougall's formula and mine agree." Fricke[7] developed his formula further to take into consideration the shape of the blood cells and considers them as oblate spheroids with the ratio of axes = 4.25. Gram[8] determined the conductivity of blood using direct current and compared these results with that of Maxwell's formula, considering the cells as insulating spheres, and found the resistance greater than that calculated from the formula. His measurements were made instantaneously.

EXPERIMENTAL.

Since the alternating current method of Kohlrausch has been used almost exclusively for the determination of the conductivity of electrolyte solutions for many years, the direct current methods have been neglected and it was necessary to spend considerable time in finding a method that would work. In several physical laboratories it has been the custom to determine the potential between two points in a solution by determining the potential difference between two platinum wires inserted at these two points. This could be checked by reversing the wires to see if the same result was obtained. A similar method was first tried with blood and some other tissues. The conductivity vessel shown in Fig. 1 was first used. It consisted of two amalgamated zinc electrodes in concentrated solution of zinc sulfate connected by three-way stop-cocks with a horizontal tube containing the blood. The

[6] Fricke, H., *J. Gen. Physiol.*, 1924, vi, 375.
[7] Fricke, H., *Physic. Rev.*, 1925, xxiii, 575.
[8] Gram, H. C., *J. Biol. Chem.*, 1924, lix, 33.

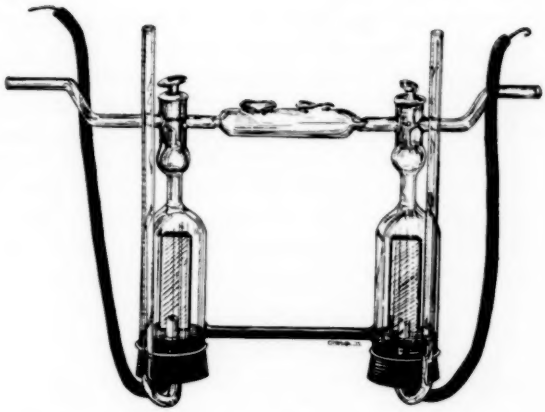

FIG. 1. Conductivity vessel for direct current, consisting of two amalgamated zinc electrodes in zinc sulfate solution connected by 3-way stopcocks to a horizontal tube containing blood. The current passing through the apparatus was measured as well as the potential difference between two platinum wires inserted in the openings of the horizontal tube.

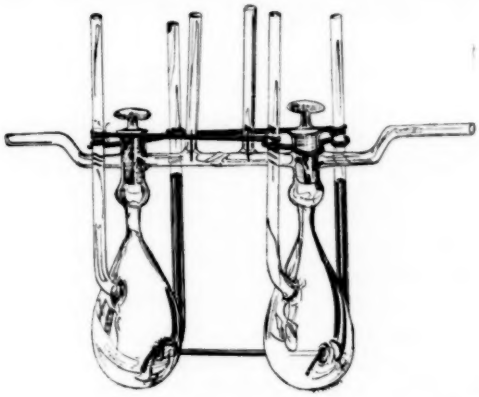

FIG. 2. Conductivity vessel similar to Fig. 1 except that calomel electrodes are used and the platinum wires are sealed in.

apparatus was immersed in a thermostat filled with oil. In the case of solid tissues, a disc of the tissue was inserted in the midpoint of the tube. The total current passed through the apparatus was measured by means of a Rawson multimeter. This instrument is a sensitive milliammeter whose range can be varied by simply turning a switch. The potential difference between two points in the blood in the tube was determined by inserting two platinum wires in the two openings at the top of the horizontal tube, the platinum wires being fixed a certain distance apart in a rigid frame. The wires could at any time be withdrawn and put in in the reverse order. The potential difference between the wires was measured by means of a Dolezalek electrometer with a platinum sputtered quartz suspension giving one division of the

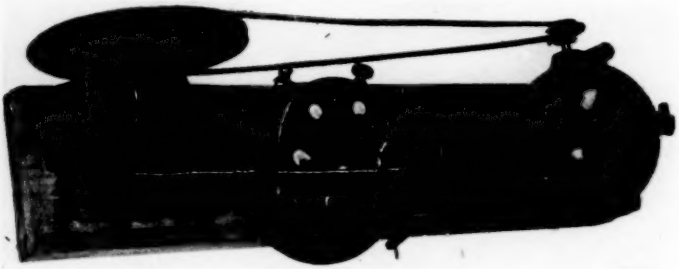

FIG. 3. Motor-driven commutator for reversing the current every 30 seconds.

scale per millivolt. This instrument was in a grounded shield and the key was well shielded. It was found impossible to make very accurate determinations with this apparatus due to the fact that the electrometer had a large capacity so the apparatus shown in Fig. 2 was made. In this case the zinc electrodes were replaced by large calomel electrodes and the platinum wires were sealed in. In order to reduce polarization at the calomel electrodes and to correct for any differences in the platinum wires, an electric motor was used to drive a commutator which reversed the current every 30 seconds (Fig. 3). After a rough determination was made a final adjustment could be sharpened during a 30 second interval and a final reading could be verified during a 30 second interval. With this apparatus some difficulty

was experienced by slow mixing of the blood with the Ringer's solution saturated with calomel which filled the calomel electrode vessel, so the electrode shown in Fig. 4 was made. In this apparatus the platinum wires were made further apart to increase the accuracy, and the calomel electrodes were above the level of the blood so that no blood corpuscles could fall by gravity into the calomel electrodes. The results were a little better but not wholly satisfactory. On the assumption that some of the trouble might be due to diffusion of the blood into the Ringer's solution in the calomel electrode, the hole in the stop-cock was filled with an agar gel made up in Ringer's solution. Since the resistance of the solution in the calomel electrode was very small compared with that of the blood in the narrow tube it was thought advisable

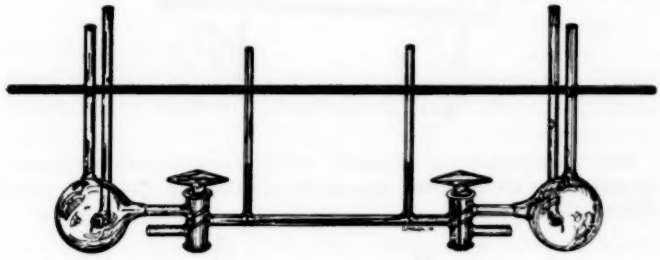

FIG. 4. Conductivity vessel similar to Fig. 2 except that the calomel electrodes are above the level of the horizontal tube.

to do away with the platinum wires and measure the difference in potential between the mercury in the two calomel electrodes and assume that it was the same as the difference of potential in the blood at the points where the Ringer's solution in the agar gel came in contact with it. For this purpose the apparatus shown in Fig. 5 was constructed. At the top of each calomel electrode vessel there was inserted a hollow glass stopper with a horizontal fused-in glass tube. At right angles to the insertion of the tube a hole was bored in the hollow stopper. The remaining hollow space in the stopper was filled with agar gel made up in Ringer's solution. There was another hole in the neck of the calomel electrode vessel to allow escape of Ringer's solution when inserting the stopper. This was closed after the stopper was inserted. The

two stoppers were placed so as to connect the fused-in tube with the horizontal tube to be filled with blood, also with a side neck ending in a funnel on one side and an overflow tube on the other.

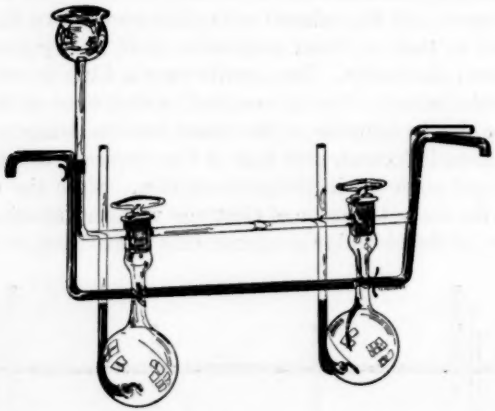

FIG. 5. Conductivity vessel similar to Fig. 2 except that the stoppers of the stop-cocks are filled with agar gel to prevent mixing of the blood and KCl solutions.

FIG. 6. Conductivity vessel similar to Fig. 5 except that a horizontal tube is in the shape of a U and can be placed in the centrifuge.

Blood was placed in the funnel and allowed to run through the horizontal tube, displacing all the air bubbles or liquid from previous determinations. Then the stoppers were turned through a

right angle, connecting the agar gel with the blood in the horizontal tube. The difference in potential between the mercury in the calomel electrodes was determined by means of a Leeds and Northrup potentiometer and the total current flowing was determined by the multimeter. The resistance of the blood was determined by Ohm's law, $I = \frac{E}{R}$ in which I is the current in amperes, E is the electromotive force in volts, and R is the resistance in ohms. In this apparatus, after determining the resistance of the blood to direct current, an alternating current could be used by inserting the conductivity vessel in a Wheatstone bridge arrangement. In order to make determinations on very high concentrations of corpuscles the conductivity vessel was modified to the form shown in Fig. 6. In this case the tube containing the blood was U-shaped and could be placed in a centrifuge to pack the corpuscles further.

Since Newbery[9] had claimed that he could make more accurate determinations with direct current than by the Kohlrausch method, using alternating currents, a modified Newbery apparatus was made as shown in Fig. 7. The apparatus consisted of two large calomel electrodes and above them were two connecting vessels filled with agar gel made up in Ringer's solution. The blood was contained in a horizontal tube, the two ends of which were inserted in the two connecting vessels, fitting into ground glass joints. When the blood tube was fully inserted, that is when the joint was tight, a capillary tube not filled with agar gel reached almost to the open end of the blood tube. This capillary tube was filled with Ringer's solution and connected to a small calomel electrode. Around the open end of this capillary tube was wound a platinum wire which served as an electrode for the alternating current for comparative measurements. When the apparatus was used for direct current, a current was passed through the blood from the large calomel electrodes and determined by the multimeter. At the same time a potential difference at the two ends of the tube containing blood as picked off by means of the capillary tubes and small calomel electrodes was

[9] Newbery, E., *J. Chem. Soc.*, 1919, cxiii, 701. Eastman, E. D., (*J. Am. Chem. Soc.*, 1920, xlii, 1648) concludes that Newbery was in error nearly 0.7 per cent and Kohlrausch's measurements correct.

determined by means of the potentiometer. The determinations with alternating current required a large inductance inserted in series with the circuit which passed through the blood by means of the platinum wires in order to produce resonance. This inductance had to be so large because the series capacity at the surface of the platinum wires was so small, but this part of the technique will be considered in the second paper which concerns itself with alternating currents.

In these determinations there was always found to be a change in resistance with time. Since the apparatus was in all cases immersed in a constant temperature oil bath and the tube contain-

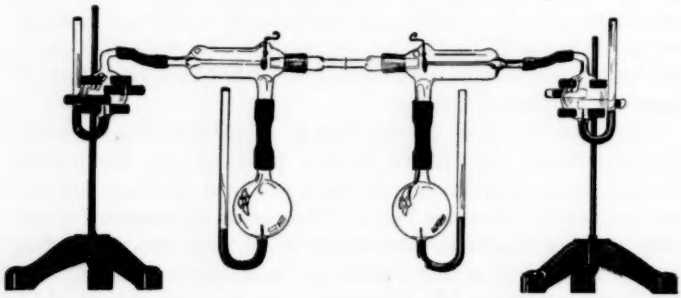

FIG. 7. Modified Newbery conductivity vessel for blood. The current is run through two large calomel electrodes below, and the voltage is picked off by two small calomel electrodes at the two ends. Two platinum loops are used to pick off the current when using the Kohlrausch method. The segments in which the platinum loops and capillary tubes from the small calomel electrodes are inserted, are filled with agar gel.

ing the blood was very narrow, and with a large surface to the oil which was circulated at a rapid rate, it was concluded that temperature could not account for this difference. In all my work for the past 15 years on the conductivity of cell suspensions, I have taken into consideration the effect of settling of the cells, contrary to the objections raised by Brooks.[10] Since these determinations took some time, and although by reversing the stopcocks the blood could be stirred up in some of these electrodes, there was some opportunity for settling. Beef corpuscles, how-

[10] Brooks, S. C., *J. Gen. Physiol.*, 1925-26, viii, 349.

ever, were used exclusively as they do not settle as rapidly as human corpuscles; in fact, they settle very slowly under most conditions. Another change which may affect the conductivity is change in shape of the corpuscles. Crenation may occur in corpuscles suspended in salt solution without any change in salt concentration as shown by Brinkman and Van Dam.[11] Crenation may occur fairly rapidly in the corpuscles in contact with a cover-glass under a microscope and if this cover-glass is passed through a flame previous to placing it over the corpuscles, the crenation is inhibited. Therefore, it was concluded that the crenation was due to an electric charge on the cover-glass which was removed by the ionized gas or electron emission in the flame. If an electric charge on the glass may produce crenation, it seems possible that the passage of an electric current under certain conditions may produce crenation, although this would probably occur only with corpuscles in salt solution.

Another change seemed, however, to be the basis of the change in conductivity, and that was the orientation of the corpuscles with a long axis parallel to the current lines. Such an orientation has been observed by other workers and this would be progressive with time so that the resistance would decrease from the instantaneous resistance and approach a maximum, which would be arrived at when the corpuscle was rotated as much as it had room to rotate in the suspension. It would therefore rotate more in a dilute suspension than in a concentrated one. Since some time was required for accurate determinations, the measurements made were the final values; that is to say, the rotation had approached a maximum so there was little change with time. The results are shown in Fig. 8 in which the resistance of the blood is recorded in units such that the resistance of the serum in the same conductivity vessel is equal to 1, or in other words, the specific resistance of the blood is divided by the specific resistance of the serum and this measured on the abscissa in Fig. 8. The cell volume in percentage of total volume of the suspension is measured on the ordinate. The cell volume in a precipitate of blood cells taken from a large centrifuge was determined by means of a hematocrit rotating at a speed of about 20,000 revolutions in a minute (centrifuged to translucence). This cell precipitate was

[11] Brinkman, I. R., and Van Dam, E., *Biochem. Z.*, 1920, cviii, 35.

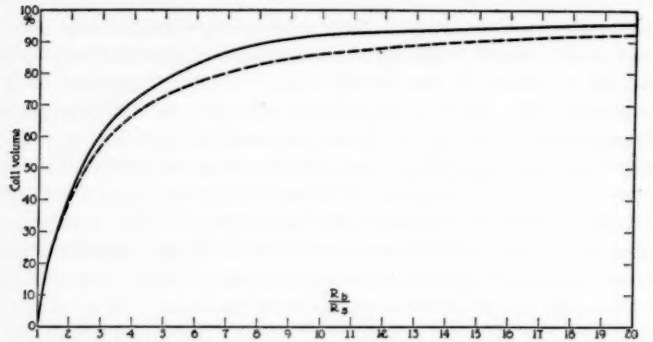

FIG. 8. Graph showing the per cent cell volume on the ordinate and the resistance of blood on the abscissa regarding the resistance of serum as unity. The smooth curve represents the resistance of blood as measured and the dashed curve as that calculated from Clerk Maxwell's formula assuming the corpuscles to be spheres of infinite resistance.

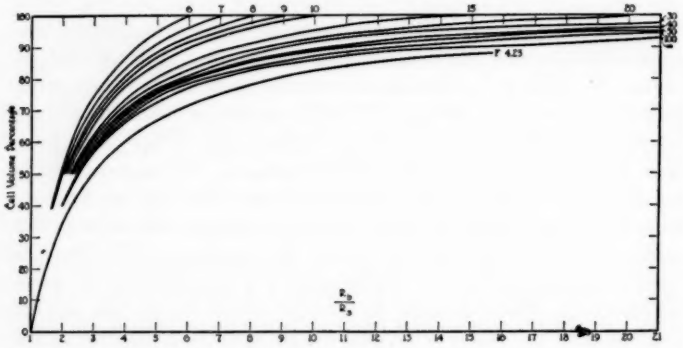

FIG. 9. Graph of calculated curves similar to Fig. 8. The lower curve marked F 4.25 is from Fricke's calculations assuming the resistance of the corpuscles to be infinite and corpuscles to be oblate spheroids with a ratio of axes = 4.25. The remaining curves were calculated by F. Fetter using Maxwell's formula. The ratio of the specific resistance of the corpuscle substance to that of the serum from 6 to infinity is marked at the end of each curve.

then kept stirred to keep it uniform and its specific gravity determined as well as the specific gravity of the serum by means of the pycnometer. The various cell volumes were obtained by weighing corpuscle precipitate and serum and mixing, since the weight can be determined much more accurately than any volumetric measurements.

The continuous curve in Fig. 8 shows the results of measurements and the dotted curve shows the calculations made from Maxwell's formula assuming the corpuscles to be spherical insulators. For an equal cell volume, the resistance of the blood is less than calculated by Maxwell's formula. The determinations made by Gram show the resistance of the blood to be greater than calculated by Maxwell's formula. The difference between these two sets of determinations probably arises from the fact that Gram made his determination instantaneously, in which case no time for orientation of the corpuscles was given, and present determinations were made after orientation had occurred. In Fig. 9 are given a number of curves using Maxwell's formula and assuming various specific resistances of the corpuscles from 7 to infinity, and also it shows the curve using Fricke's formula, assuming the diameter of the corpuscle to be 4.25 times its thickness and its resistance infinite. Fricke's formula probably harmonizes more with Gram's data, but the present determinations more with Maxwell's formula, assuming a slight conductivity of the corpuscles. Since Fricke has shown that the ellipsoid shape of the corpuscles increases the resistance when they are at random arrangement over that which they would have if they were spheres, and has emphasized also that orientation will decrease the resistance, the orientation acting in an opposite direction from the effect of flattening of the corpuscles on the resistance, one would expect the present determinations to approach more toward Maxwell's formula. Since it is well known that chlorine ions may go in and out of the corpuscles, it is safe to assume them to have a slight conductivity. The passage of ions must be very slow, however, or else we would not get the relatively high resistance of corpuscles which has led many investigators to assume that they were insulators. Since the results with alternating current shed further light on this question, a discussion of it is reserved for the second paper of the series.

VITAMIN E AND REPRODUCTION ON SYNTHETIC AND MILK DIETS.*

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(Received for publication, March 19, 1926.)

The reproductive behavior of rats on various diets in which milk is the only source of proteins and of vitamins is strangely variable. When such diets are made rich in fat, either by the use of high percentages of whole milk powder or by the inclusion of lard, sterility ensues in the first or second generation; when they contain no other fat than that which is supplied by a lower percentage (40 to 50) of milk powder, reproduction is successful (1–3). The sterility which we observed on high fat, milk rations (4) was, in the case of female animals, of the same type as that described by Evans and Bishop (5) as a result of vitamin E deficiency and consists in the death and resorption of the implanted and developing embryos. It is promptly corrected by including sources of vitamin E in the diet. Male animals deprived of vitamin E also become sterile, as we demonstrated in 1924 (4), but fertility is not interfered with until they have been on a high fat, milk diet up to 3 to 5 months of age. Within this period there is initiated a degeneration of the seminal epithelium which, when advanced, is rarely, if ever, curable by administration of vitamin E. It is, however, preventable if sources of vitamin E are supplied before 3 months of age and thereafter. Such a degeneration has often been described as a result of inadequate vitamin B in the diet but the milk diets used were not deficient in this accessory.

In view of the marked differences exhibited by animals on the two types of milk ration and of the apparent dissimilarity of the

* Presented in part before the American Society of Biological Chemists, December, 1925 (Mattill, H. A., and Clayton, M. M., *J. Biol. Chem.*, 1926, lxvii, p. xlix).

sterilizing effect in the two sexes, it seemed necessary to determine whether the same degeneration of the testes would supervene on synthetic diets lacking known sources of vitamin E and whether the amount of fat in such diets had a modifying influence comparable to that found with milk diets. Also, in view of the hesitation of some investigators (6) to accept the existence of vitamin E as proven, we desired some original evidence as to the efficacy of yeast in preventing sterility when it was used as a source of vitamin B in synthetic rations.

TABLE I.
Rations.

Ration No.	Casein.	Starch.	Lard.	Salts.*	Cod liver oil.	Wild yeast.	Molasses yeast.	Other additions.
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
195	18	48	23	4	2	5		
196	18	48	23	4	2		5	
197	18	63	8	4	2	5		
198	18	63	8	4	2		5	
199	18	67	8	4	2			Vitamin B Harris 1.
201	18	63	3†	4	2		5	Wheat germ oil 5.
202	18	63	3†	4	2		5	Cottonseed oil 5.
203	18	63	3†	4	2		5	Olive oil 5.
205	18	63	7.5†	4	2		5	Unsaponifiable constituents, cottonseed oil 0.5.
206	18	63	7.8†	4	2		5	Unsaponifiable constituents, olive oil 0.2.
207	18	63	8†	4	2		5	

* Salt mixture of Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.* 1917, xxxii, 317.

† Lard rendered below 60°C.

Experiments with Synthetic Rations.

About 150 animals taken at weaning were reared on the rations indicated in Table I. The casein was a commercial product which we did not refine in any way. Two kinds of yeast were used,[1] a wild variety and a molasses yeast. Each of these was incorporated in a high fat and in a low fat diet containing 23 per cent

[1] The yeast used in these experiments was kindly supplied by the Fleischmann Company.

and 8 per cent of lard, respectively. A fifth group of animals was given vitamin B Harris instead of yeast as a source of vitamin B. A sixth group was given the low fat ration with 5 per cent wheat germ oil[2] replacing 5 per cent of lard, and four other groups were given 5 per cent of cottonseed oil, olive oil, or a generous equivalent of their unsaponifiable constituents.[3]

In several of these rations commercial lard was replaced by special lard rendered in the laboratory at low temperature, not exceeding 60°C., and one further lot of animals was fed such a

TABLE II.

Ration No.	No. of ♀ animals.	Resorptions observed.	Births.	Young born.	Young survived.	No. of ♂ animals.	Sterile.*
195	6	8	0			8	8
196	9	9	0			9	9
197	7	6	0			7	7
198	10	4	0			8	7
199†	6					9	9
201	5	0	11	56	14	8	0
202	5	7	0			6	5
203	5	5	1	4	0	7	6
205	3	5	0			1	1
206	2	3	0			2	2
207	8	4	1	2	0	9	6
207r‡	5	6	1	3	0	8	6

* After curative experiments (except for Rations 199 and 207r); these animals are again discussed in connection with Table III.

† Due to the effects of a temporary shortage of vitamin B in this ration the animals on it have been omitted from the discussion.

‡ Radiation for 10 minutes on alternate days with Hanovia Luxor lamp at 3 feet. Half of the animals were radiated, the other half received the ration after it was radiated.

ration after radiation with ultra-violet light or the animals themselves were so radiated (Ration 207r, Table II.)

[2] Extracted from wheat germ by anhydrous ether.

[3] The unsaponifiable constituents of these oils were obtained by repeated hot alcohol extraction of the oils. The residue after evaporation of the alcohol was extracted with ether. The extract was saponified by 20 per cent alcoholic KOH at 30°C. and extracted with ether. The crystalline material remaining on evaporation of the ether amounted to about 0.5 per cent of the original oil and was incorporated directly into the ration or, later, made into pellets with lactose and fed separately.

Good growth obtained on all the rations except the one containing Harris vitamin, and inasmuch as substitution of 5 per cent of yeast caused resumption of growth we assumed that for some unknown reason the concentrate had lost its efficacy. At the time there was no opportunity to look into the matter further. The animals appeared sleek and well nourished but, as seen in Table II, there was no reproduction on any of the rations except on the one containing wheat germ oil. On all the others resorptions were common and later these ceased to appear. This is quite in line with our previous observations (4); the males are at first fertile, even in the absence of vitamin E, but subsequent degeneration of the testes eliminates the possibility of resorptions in the females. The three births recorded under Rations 203, 207, and 207r are typical of the deceptive character of the data sometimes obtained. The first litter in each case was the only one, the next was resorbed. Apparently enough vitamin E remained from the preexperimental weaning period to carry the animal through one gestation but not beyond one. Evans has very properly called this behavior "first litter fertility" and it is another instance of the necessity of considering the nature and extent of the reserve stores with which an animal enters upon a period of experimental feeding.

On the ration containing wheat germ oil all the females bore young repeatedly and at autopsy the male gonads were found to be of normal weight or above, in contrast to those of the males on all the other rations. The testes weights of the latter showed that all of them, with a few exceptions among the younger animals, had suffered more or less complete degeneration. The data will be discussed later in connection with the curative experiments.

The facts which we established on male rats on high fat, milk rations are thus abundantly confirmed on synthetic rations and they have recently also been confirmed by the observations of Evans (7) and of Mason (8).

No difference could be noted between the effects of the two varieties of yeast. Neither contains vitamin E and the distinction between them was abandoned in the latter part of the experiment.

In view of the facts established by others (9, 10) the ineffectiveness of cottonseed and olive oils in inducing reproduction in our tests is to be accounted for by their low concentration in the diet.

They cannot be considered equal to wheat germ oil but possess a much smaller amount of vitamin E.

Curative Attempts with Male Animals.

With the relation of vitamin E to the reproductive function in male animals established for synthetic rations it was important to determine by curative tests whether the damage done by its absence was as permanent as it appeared to be in animals on high fat, milk diets. Accordingly there was incorporated into Rations 195 to 198 1 per cent of wheat germ oil for some animals; later for some of these and for others 7.5 per cent of unextracted wheat germ (containing less than 1 per cent of oil) was added. Male animals on Rations 202 to 206 were placed directly on the colony ration and ultimately most of the males were given this food which consists of the McCollum stock diet, biscuits (Castle's formula),[4] with green lettuce leaves and raw liver once a week. These transfers took place at various ages for the individual rats, and the males were mated at intervals with colony females of known fertility.

The results of these tests are summarized in Tables III and IV and no tests are included unless the female bore young from a normal mating immediately preceding or following the test. The last two columns show respectively the duration of the curative period and the weights of the testes at autopsy, the latter expressed in per cent of the expected for the body weight according to Donaldson's table. Among the thirty animals from Rations 195 to 198, which were submitted to these tests, only one gave a positive response. This animal received a 1 per cent addition of wheat germ oil at 90 days of age, was infertile in three successive trials at 115, 150, and 170 days of age, was placed on the colony ration at 190 days of age, and 125 days later (over 7 months after vitamin E feeding began) sired 14 young. The weight of the testes at autopsy, 53 per cent of the expected normal, is the lowest weight for a fertile animal which we have encountered. Of the twenty-two males originally on Rations 202 to 207 and transferred to the colony ration five proved to be fertile. With three exceptions all of the twenty-two were less than 150 days old when transferred. Unfortunately these animals could not be tested functionally at the

[4] Manufactured by Potter-Wrightington, Boston.

TABLE III.

Recovery Tests on Male Animals.

Ration No.	Rat No.	Age transferred to: Same ration plus: 1 per cent wheat germ oil.	Age transferred to: Same ration plus: 7.5 per cent wheat germ.	Age transferred to: Colony ration.	Age at fertility test. Negative.	Age at fertility test. Positive.	Age at death.	Length of curative period.	Weights of testes in per cent of normal for body weight (Donaldson).
		days	*days*	*days*	*days*	*days*	*days*	*days*	*per cent*
195	4293		132	207	115, 130, 160, 180, 330		334	202	46
	4269	118		215	140, 180		316	198	34
	4275	116		213	140, 180		314	198	40
	4242		153	228	125, 145, 185, 195		319	166	31
	4235	125		225	150, 185		323	198	37
	4211				160		186	41	41
	4256		150		130, 145, 180		191		55
	4218						101		53
196	4257	151		222	125, 145, 180, 420		430	279	43
	4308	90		190	150, 400		412	322	30
	4246	125	153	228	150, 185, 200		323	198	40
	4224	182		250			351	169	31
	4212		187				216	29	50
	4236		152	227	135, 150, 190, 200		305	153	28
	4270	118	153	222	145, 175, 190, 320		331	178	27
	4282	115		215	140, 175		328	213	35
	4299						130		46
	4219				160, 180		185		31
197	4283	115	143	218	140, 180, 195, 410		423	308	46
	4273		144	219	175, 195, 320, 410		424	280	32
	4294	132		204	165, 325		334	202	29
	4258	150		222	185, 315		320	170	69
	4220	185		257	160, 180, 215		355	170	49
	4249	123	151	225	150, 185, 205		321	198	32
	4271	118		205	140, 180		331	213	34
	4239						153		55
	4213		186		160, 190, 213		216	30	63
198	4300		130	205	175, 305, 400		411	281	40
	4307	90		190	115, 150, 170	315 (14)*	319	225	53
	4312	118		190	150, 170, 310		319	201	28
	4284	115		215	140, 180		345	230	31
	4262	120	148	224	145, 200		316	196	40
	4274	116		216	125, 145, 175		314	198	35
	4230		177	252	150, 175, 230		347	170	27
	4250	152		223	130, 150, 185, 315		321	169	38
	4240	125			130, 150, 180		182	57	54

* The figure in parenthesis is the number of young born.

TABLE IV.

Recovery Tests on Male Animals.

Ration No.	Rat No.	Age transferred to colony ration.	Age at fertility test.		Age at death.	Length of curative period.	Weights of testes in per cent of normal for body weight (Donaldson).
			Negative.	Positive.			
		days	*days*	*days*	*days*	*days*	*per cent*
202	4333	159		300 (12)* 315 (4) 320 (10) 322 (7)	351	141	**71**
	4404	136	235, 330		344	108	40
	4330	159	260		272	113	39
	4394	140	235		238	98	31
	4359	148	265		278	130	55
	4377	143	260		273	140	37
203	4395	140		258 (7) 290 (8) 300 (7) 307 (4)	348	118	**90**
	4405	136	240, 330		344	208	56
	4349	148	230, 340		356	208	31
	4378	143			256		47
	4331	159	250		257	98	51
	4347				262		35
	4336	159			288		40
205	4379	143	260		273	130	35
206	4396	141	255		270	129	34
	4386	141	260		271	130	28
207	4448	96	200, 290		304	208	34
	4387	141	265, 290, 340		349	208	35
	4426	122		240 (8) 280 (4)	314	118	61, **116**
	4436	118		215 (12)	248	97	**72**
	4408	133		230 (8)	246	97	**72**
	4383	143	240		252	109	36
	4362	146	266		276	130	39
	4356	148	265		274	126	29
	4368	140	240		244	104	46
207r	8♂ animals.		Negative with ♀ on same ration.		143 to 169		51, 77, 51, 50, 46, 53, 49, 94
201	8♂ animals.			Continuously fertile with ♀ on same ration.	151 to 180		115, 105, 114, 87, 94, 98, 104, 102

* The figures in parentheses are the number of young born.

time of transfer and it is therefore impossible to say whether or not degeneration in the five fertile animals had ever proceeded to the point of infertility. It certainly had in the other seventeen and some of them were younger than the subsequently fertile animals at the time when the ration was changed. However, to consider the fertile animals as instances of recovery is not justifiable for we cannot exclude a possible protection and intact survival of portions of the seminal epithelium.

As one among thirty animals to show recovery this represents a much smaller success (only 3 per cent) than Evans (7) has recently reported (20 per cent). His animals were 5 to 8 months of age when the cure was begun; ours, as appears in the table, were mostly younger and the curative régime lasted in most cases from 4 to 8 months, in some cases much longer. His success in achieving cures in 4 to 6 months may be due to the administration of wheat germ oil separately rather than depending upon the food intake to determine the amount of vitamin E which the animals obtained.

It is impossible to find any relation between the length of the curative period and the state of the testes as indicated by their weight and by the records of test matings. Thus ♂ 4307 on Ration 198 and the litter mate ♂ 4308 on Ration 196 had exactly the same treatment. The former became fertile after 225 days, the latter failed to become so after 322 days of the same curative régime. A similar absence of correlation exists among the animals on Rations 202 to 207.

The age at which the curative régime is begun is of paramount importance. In these series 159 days is the latest date at which it was begun with ensuing success but as in the instance cited above and in many others it was begun much earlier without ultimate success. Since, in the great majority of cases, restoration is impossible, it seems reasonable to conclude that true restoration is never possible and that apparent cures represent undegenerated tubules whose function has been partially maintained.

While vitamin E is thus very necessary in the period of early maturity, it can be removed from the ration of an animal later in life without such immediate consequences. Data obtained in a study of high fat, milk rations (11) suggest that if vitamin E is supplied in adequate amounts for the first 90 days of life it may be

dispensed with thereafter for a longer period. From a lot of twenty-one animals reared on the colony ration individuals were transferred at various ages to the high fat, milk ration which always causes sterility. After various intervals the animals were sacrificed. The weights of the testes at autopsy expressed in percentage of the expected weights as given in Chart 1 showed that of eleven animals transferred before 95 days of age only two escaped degeneration. Of ten transferred after 95 days of age only three suffered degeneration within 72 to 147 days. The converse of this test was also carried out with high fat, milk rations. Of sixteen animals transferred from the milk to the colony diet after 95 days of age only two escaped degeneration.

Mason's (8) deprivation experiments on older animals were begun when they were 60 to 105 days of age and showed the critical period on the vitamin E-deficient diet to be 75 to 100 days, several weeks longer than for animals taken at weaning. While there is no evidence that an older animal can entirely dispense with vitamin E yet it would seem that the longer an animal is carried on a complete ration the longer the critical time on a subsequent deficient diet is postponed both absolutely and relatively. This is doubtless due to the gradual accumulation of vitamin E in the tissues. Possibly the rate of exhaustion is lower in male animals than in female (10).

The validity of using the gross weight of the testes as a criterion of their normality may be questioned. From the figures on fertile animals printed in heavy type in the last column of Tables III and IV it is evident that with one exception no organs below 70 per cent of the expected weight were functional. From data secured on colony males of all ages we had provisionally adopted the figure of 80 per cent of the expected weight as being the lowest limit compatible with normal function. Of forty-two animals examined at 120 to nearly 500 days of age, three fell below the figure of 80 per cent; one was 78 per cent, the other two were below 60 per cent. The figure of 70 per cent of the normal weight is perhaps a fair but not infallible index of fertility.

It is probably as good an index as microscopic examination can offer. The state of one gland removed for sectioning is not entirely trustworthy evidence for the state of the other. If complete serial sectioning of one gland should show the entire

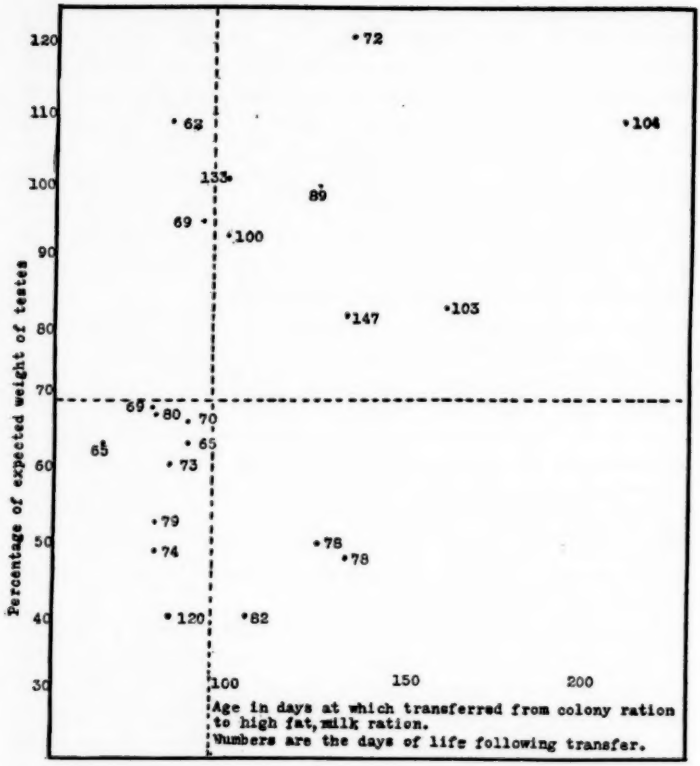

CHART 1. The ordinates are the relative weights of the testes at autopsy expressed as percentage of the expected figure for the animal's body weight, according to Donaldson. The abscissæ are the age in days at which the animal was transferred from the adequate colony diet to a high fat, milk ration which produces sterility. The adjacent figures indicate in each case the number of days the animal lived on this inadequate diet thereafter. Of eleven animals transferred before 95 days of age only two escaped degeneration. Of ten animals transferred after 95 days of age only three suffered degeneration within periods of 72 to 147 days. The critical age is about 90 days. If vitamin E is supplied throughout this growing period an animal may dispense with it for an even longer time thereafter.

absence of sperm, the remaining organ might still possess some as yet undegenerated tubules. Indeed we have found several instances of one entirely normal and one markedly degenerated gland in the same animal. One of these was the fertile male Rat 4426, Ration 207 (Table IV). Only a functional test can be relied upon to give accurate information upon procreative power.

It is of interest that when fertility was retained by male animals whose organs showed some degeneration, the size of resulting litters was not diminished. This agrees with the observation made by Parkes and Drummond (12) on animals deprived of vitamin B. In their words, if the animal is potent at all, as many ova can be fertilized as are offered by the female.

The recent observations of Parkes and Drummond (12) just referred to confirm earlier ones made by Drummond and by many others to the effect that a shortage of vitamin B causes male sterility. Apparently this may be produced at any age in a very short time if vitamin B is completely withheld. Degeneration of the testes has been recorded under various other dietary deficiencies but in no case has this been the only indication of an inadequacy in the diet. In vitamin E deficiency there are no outward marks; the animals manifest all the signs of health and well being. The relationship, if any, between vitamins B and E in the matter of fertility of the male animal deserves inquiry. This question and also the chemical composition of degenerated gonads as compared with the normal organs we are studying at present.

Curative Results with Female Animals.

The prompt restoration of fertility in female animals when sources of vitamin E were included in the high fat, milk rations led us to believe that a similarly prompt recovery would follow the introduction of vitamin E into the synthetic rations on which they had been sterile. Indeed the very extensive work of Evans and Bishop (5) made our experiments almost unnecessary, except that no series of recovery tests seems to have been published in confirmation of this work. Also we expected to find that the amount of fat in synthetic rations had a modifying influence. Unfortunately the series is not complete because of an inadequate number of animals. Table V shows the percentage of response

and the period within which it appeared upon the addition of 1 to 4 per cent of wheat germ oil to the basal rations. The percentage response is a gradually ascending series in the order: 1 per cent wheat germ oil in the high fat basal ration—1 per cent in the low fat basal ration—2 per cent and 4 per cent, the latter two in low fat basal rations. The time elapsing between the first administration of wheat germ oil and the birth of the first litter

TABLE V.

Curative Results with Female Animals.

Addition to basal ration.	No. of animals.	Positive responses.	Average days to birth after addition.	Average no. of young.	Average no. reared.
1 per cent wheat germ oil high fat	8	3 (38 per cent).	97	2	0
1 per cent wheat germ oil low fat	9	5 (55 " ")	60	3	0
2 per cent wheat germ oil low fat*	15	11 (74 " ")	42	7.5	0.8
4 per cent wheat germ oil low fat	7	6 (86 " ")	26	5	
7.5 per cent wheat germ (less than 1 per cent of oil)†	14	13 (93 " ")	47	6	3.0
Fed from weaning the basal ration with 5 per cent of wheat germ oil (Ration 201)‡	5	5 (100 " ") 11 litters.	95 days age at first litter.	5	1.3

* Half the animals on the 2 per cent addition had a basal ration containing special lard rendered at low temperature.

† Both high and low fat rations.

‡ From Table II for comparison.

is a gradually decreasing quantity for the same series of oil additions from 97 to 26 days. The differences in behavior between the animals receiving the 1 per cent oil additions to the high fat and the low fat basal ration are perhaps no more than might be expected on the basis of a smaller intake of a high calorie diet and the resulting smaller amount of wheat germ oil consumed. An attempt at correlation is complicated by the presence of the time

factor but this behavior is at least in marked contrast to that of animals on milk rations high in fat and on those low in fat (4, 13). Here there was a marked and qualitative difference; low fat rations allowed reproduction, high fat rations did not. On these synthetic rations there is a fairly linear relationship between the amount of vitamin E supplied and the promptness with which its functional result is achieved.

When 7.5 per cent of unextracted wheat germ containing less than 1 per cent of oil was added to the basal ration, the difference in favor of the low fat ration was so slight as to be negligible and the two sets of figures are combined in the table. They indicate a higher percentage of responses than was secured with even 4 per cent of the extracted oil and within a period of time approximating that found with 2 per cent of oil. If there is a difference between the high fat and low fat rations in the amount of wheat germ required, the amount used was too large to expose it.

The number of young per litter did not approach the normal with 1 per cent of oil. Above that, and with 7.5 per cent of wheat germ, it was approximately normal. Lactation was entirely inadequate and showed only very slight improvement with increasing amounts of oil. Unfortunately observations on the young born on the 4 per cent addition had to be discontinued before survival data were secured but inasmuch as animals which had from weaning received 5 per cent of wheat germ oil reared only 25 per cent of their young, it is not possible to deduce from these data any relationship between vitamin E and lactation. Some other limiting factor must be involved; for its absence from the basal ration there is apparently some compensation in the unextracted wheat germ since animals on this supplement reared 50 per cent of their young. One may suspect this factor to be vitamin B (9) although all rations contained 5 per cent of yeast. It may be traces of mineral matter (14), bulk (15), or some as yet unknown substances (16) occurring in natural foods.

A temporary but marked acceleration of growth took place in animals of both sexes when wheat germ or wheat germ oil was added to the basal ration whether fertility was subsequently evoked or not.

DISCUSSION.

The advent of vitamin E as a dietary constituent necessary for the foundation and maintenance of normal functions throws into relief some new lines in the structure of our nutritional conceptions thus far erected. One of these is the sex-different response to its absence—a qualitative difference more distinct than has been indicated for any other food accessories. Female animals deprived of vitamin E can apparently resume their inhibited procreative capacity at any time in the reproductive period if vitamin E is provided. Function suffers but the organ structure required for the process does not. In males the latter is destroyed beyond a certain critical age. However, the amount of vitamin E that is required for bringing the testes to their normal mature structure is no more than a young animal from a colony has accumulated in his tissues at the time of weaning. That this store is responsible for the maturation of the testes when the diet does not contain vitamin E may be deduced from the observations of Evans (7) upon young male animals whose mothers for one or two generations had been kept on rations devoid of E, temporary fertility being induced by administering minimal amounts of vitamin E concentrates. Such males were sterile from the beginning of sexual maturity. In a few instances of such second generation animals on high fat, milk rations, we found degeneration appearing much earlier than it normally does.[5] The small amount of vitamin E required to bring the testes to maturity at 2 to 3 months of age on a ration devoid of vitamin E probably explains the considerable variability in the time of incidence of sterility among different animals on the same deficient ration. An increase in this store of vitamin E in properly nourished animals probably explains their ability to suffer deprivation in later life without such prompt damage to the organs.

In their description of the degenerated organs of males deprived of vitamin B Parkes and Drummond (12) mention a hemorrhagic and bloodshot appearance which we have often observed in the testes of animals deprived of vitamin E. A relation between the two degenerative processes might have a common basis in a purely vascular derangement. Before assigning to the same cause

[5] Mattill and Stone (2), p. 450.

the uterine failure and fetal resorption in the female it is necessary to consider the work of Long and Parkes (17). They found resorption to be a process of autolysis of the dead fetus, which began in the liver. The process could be duplicated *in vitro* under sterile conditions if the products which are inhibitory were removed. All but the skeletal tissue went into solution. In the uterus this also disappears and the placentæ disappear last. The removal of the products, simultaneously, from a number of autolyzing embryos at a rate rapid enough to be reflected in the gross weight of the animal does not suggest an impaired circulation in the uterus. According to Evans and Burr (10) only the fetal parts of the placenta suffer, structurally, and the yolk sac and allantois also exhibit subnormality. If none of the maternal structures are damaged by the absence of vitamin E there is some basis for the prompt recovery of female animals following its administration as against the failure to recover exhibited by male animals.

Another new point of view suggested by vitamin E experiments concerns the effect of the nature of the diet on the amount of this accessory required to prevent sterility. A more or less obscure influence of one dietary constituent upon the requirement of another has often been assumed in explaining the results of feeding experiments. Thus it has again been claimed (18, 19) that the carbohydrate of the diet determines the amount of vitamin B required, and McCollum (20) has recently remarked upon several other complicated relationships. We concluded from our earlier experiments (4) that increasing the amount of fat in the diet raised the vitamin E requirement. In those experiments when the butter fat in a 50 per cent whole milk powder ration was the only fat and the only source of vitamin E, the animals were fertile for several generations with only occasional degeneration of the testes. On the same ration, but containing 15 per cent of lard, there was typical sterility due to vitamin E deficiency. In the curative experiments with synthetic rations described above, it did not matter whether the rations contained much or little lard. It should be borne in mind however that here the source of vitamin E was wheat germ or the oil.

Having in mind the possibility of a destruction of vitamin E similar to that suggested by Fridericia (21) respecting vitamin A,

one of the curative rations for female animals contained specially prepared lard, rendered at low temperature (see Table V) but this was not superior to the ration containing commercial lard. The presence of such lard in high fat, milk rations did not prevent sterility.[6] In order to avoid a possible destructive effect on the antirachitic vitamin which Steenbock (22) lately suggests as the explanation of Fridericia's observations, radiation tests were carried out upon animals on high fat, milk rations as well as upon the rations themselves. In neither case was fertility induced nor was it in the control animals on a synthetic diet with the same treatment (Table II). A destruction of vitamin E by lard in the ration is therefore very doubtful especially in view of the great stability of E which Evans and Burr (10) have demonstrated.

The simplest explanation of the difference between the results obtained on the two types of milk rations would be the assumption that butter fat carries a small amount of vitamin E, so small that the amount secured by a rat on a high calorie diet is inadequate. This assumption Evans properly makes as a result of his observations on high fat, milk diets but unfortunately the results on low fat, milk diets do not support it. Furthermore a survey of the literature reveals many instances of fertility, some records extending through several generations, on rations in which, according to our present knowledge, the only source of vitamin E was a relatively low concentration of butter fat (10 per cent and less). The most recent of these reports is that of Hogan and Harshaw (23) who also cite some of the earlier papers. Of interest is also the work of Hoffman (24) on Vitovim bread which contained 5.8 per cent of fat (dry basis); half of this was butter fat, the other half, undesignated, was in the other constituents, white flour, yeast, and the vitamin B extract. This latter, being an aqueous extract of defatted wheat germ, should be free from fat[7] and is so

[6] These and other data on milk rations will appear in a subsequent paper.

[7] Dr. W. M. Sperry of the Department of Biochemistry has examined a single sample of "Vitavos" (the vitamin B extract) by the Kumagawa-Suto method and finds 0.06 and 0.07 per cent of fatty acids plus unsaponifiable matter. The amount of Vitavos incorporated into the now obsolete Vitovim bread has, so far as we are aware, not been indicated, but even if as much as 10 per cent was employed, the amount of vitamin E from this source was very small.

reported (25). If it did not contain vitamin E, it is impossible to explain the continuous reproduction in rats confined to a diet of this bread and water, unless both fine white flour and butter fat contain more vitamin E than has been credited to them. Another instance is found in the work on molasses by Nelson, Heller, and Fulmer (26). Unless cane molasses supplies vitamin E, an otherwise synthetic ration containing adequate vitamin B but with only 5 per cent of butter fat permitted reproduction to the fifth generation. It is, of course, entirely possible that aqueous preparations of natural foods may carry a quantity of vitamin E that is demonstrable by the biological method even though the fat content of such preparations is vanishingly small or even nil. Evans and Burr cite experiments suggesting this possibility[8] and a fair analogy is furnished by the observations of Sherman (27) on vitamin A in milk, skim milk containing half as much of this accessory as whole milk.

Many other factors tend to complicate a synthesis of the data from many sources. Among them are a seasonal variation in the origin of the products used, notably butter fat and milk powder, and the presence of minute amounts of the minerals recently shown to be important for reproduction (14). We are inclined to the belief, however, that milk fat is not as poor a source of vitamin E as has been assumed and Daniels and Hutton (28) have recently stated a similar opinion.

It is not very helpful to suppose that vitamin E as it occurs in milk fat is more vulnerable to oxidative or other destruction than it is in other fats. Supplee and Dow (29) found that old milk powder, unless stored in deoxygenated air, was not effective for reproduction while freshly prepared milk powder was, but they do not contend that vitamin E is necessarily the variable factor. We have obtained some evidence pointing in the same direction.

We are at present more inclined to the belief that the nature of the diet and the resulting chemical and bacteriological processes going on in the intestine may determine the amount of vitamin E which is actually absorbed. Excessive putrefaction or dietary conditions leading to it have been associated directly and indirectly with sterility (30, 31). The diarrhea observed in rats on skim milk powder rations (9) and in cats fed on whole milk (32) indi-

[8] Evans and Burr (10), p. 338.

cates an abnormal intestinal condition. We have been unwilling to assume that an intestinal condition played a rôle in the sterility of rats on high fat, milk rations because in most animals on such rations the growth curve is normal. Nor have we been able to correlate a higher intestinal reduction as determined by Bergeim's method (33) with subnormal growth rate in a group of animals on high fat, milk rations caged together but showing unequal growth. But they might all nevertheless be under an intestinal handicap manifesting itself in reproductive failure, if in no other way. Possibly the improved reproduction when agar was added to rations without bulk (15, 23) is related to the correction of unfavorable intestinal conditions. Nelson and his coworkers (6) in discussing the differences revealed by rats on high and low fat, milk rations, emphasize the importance of a proper quantitative relationship between the constituents of the diet, but the marked difference observed (34) between rats on a whole milk powder ration effective for reproduction and on a skim milk powder ration identical in chemical composition but much less effective for reproduction is not to be explained merely on that basis.

The influence of yeast as an adjuvant to reproduction aside from its content of vitamin B has often been discussed. Whether it affects intestinal conditions favorably, or supplies certain essential inorganic salts (28), it certainly does not provide any significant amounts of vitamin E. Yeast may not be entirely devoid of vitamin E as it is not of vitamin A (35), and cultural conditions may also affect this factor, but there are few, if any, instances of successful reproduction on yeast-containing rations that cannot be explained by the presence of vitamin E, provided we grant that milk fat is not as deficient in vitamin E as has been claimed. Whether intestinal conditions actually modify the absorption and utilization of this accessory as found in milk fat remains to be proved, but no other hypothesis has occurred to us which suggests a possible explanation of the contradictions growing out of experiments with milk powder and butter fat as sources of vitamin E.

SUMMARY.

Observations on 150 animals on various synthetic rations indicate that vitamin E is a necessary dietary constituent for the

establishment of fertility in male rats, as was first shown in experiments with milk rations high in fat (4). Animals reared on synthetic rations devoid of vitamin E show the same critical period, from 90 to 150 days of age, beyond which the administration of vitamin E seldom restores the degenerated testes to normal function. When it does so, the cures are believed to represent the intact survival of portions of the germinal epithelium. The gross weight of the testes is a fair index of the extent of degeneration; organs less than 70 per cent of the expected normal for the body weight (Donaldson) are rarely functional. Histological examination of one gland does not reveal the condition of the other, as degeneration may not proceed equally. When older animals are deprived of vitamin E, degeneration is postponed for a longer time, probably because of the increased stores of vitamin E in the tissues.

Female animals on synthetic rations devoid of vitamin E exhibit the same type of sterility as is encountered in animals on high fat, milk rations and fertility is promptly restored by including sources of vitamin E in the diet. The larger the amount of vitamin E supplied the more prompt is the response and the greater is the percentage of animals which respond. A fairly linear relationship is established. A relationship between vitamin E and lactation cannot be made out when a synthetic ration is used which contains casein, yeast, cod liver oil, salts, starch, and varying amounts of wheat germ oil. The use of native wheat germ in place of wheat germ oil in such rations improves lactation slightly. Yeast is not a source of vitamin E.

The restoration of female animals by means of wheat germ is apparently independent of the fat content of the ration. There seems not to be any destruction of vitamin E (as found in wheat germ oil) by being incorporated with lard in a synthetic ration, as has been suggested in connection with vitamins A and D, nor does radiation compensate an animal for the absence of vitamin E from the diet. The contradictions growing out of experiments with milk rations (added lard produces sterility, while a 50 per cent milk powder ration with no added fat allows reproduction) are reflected in the many experiments found in the literature, in which low levels of butter fat provide adequate vitamin E for continuous reproduction if the diet is low in fat, but not so if the

diet contains lard. A review of several such instances leads to the belief that milk fat contains more vitamin E than has been estimated, and as a working hypothesis it is suggested that unfavorable dietary adjustments and resulting undesirable intestinal conditions may increase the requirement of vitamin E in the ration if an animal is to absorb enough to remain fertile.

Addendum.—In two very recent papers Nelson, Fulmer, and their coworkers (36) again express doubt as to the proved existence of vitamin E. Some of their objections to existing evidence have been met by the work described in the foregoing paper. In all of their synthetic rations they persistently used butter fat as the source of vitamin A, and in the light of the above discussion it is not surprising that reproduction (not necessarily also lactation) was satisfactory when the amount of vitamin B was adequate and when the diets contained no lard. When lard was included 5 per cent of butter fat was no longer adequate and their animals exhibited the typical symptoms of vitamin E deprivation, especially the "first litter fertility". They report no observations on the male gonads and they make no mention of our paper (4) of October, 1924, in which the nature of the susceptibility of male rats to vitamin E deprivation was first described.

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STUDIES OF THE METABOLISM OF WOMEN.

II. CYCLIC VARIATIONS IN URIC ACID AND TOTAL NON-PROTEIN NITROGEN CONTENT OF BLOOD.*

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The questions of the extent and type of influence exerted by the sexual cycle on the nitrogen metabolism in women have attracted some attention over a fairly long period of time. Jacobi (1) in 1876 reported variations in the amount of urea excreted at certain phases of the menstrual cycle. Unfortunately, the nitrogen intake of her subjects was not controlled, hence her findings must be considered inconclusive. Much later (1918), however, Sherman and his coworkers (2) reported on excellent analytical evidence a nitrogen retention in the period immediately preceding menstruation, and a somewhat increased nitrogen loss after the beginning of the menstrual flow. These results were, in general, confirmed by the more detailed investigation of Sakamoto (3) who considered, however, that the total rate of nitrogen metabolism was lowered during the time of the menstrual flow, and that the first few days after the cessation of bleeding were marked by the most rapid rate of catabolism and excretion. Bond (4) reported an increased output of ammonia, suggesting, perhaps, an alteration in metabolism which necessitated an increase in the excretion of acidic substances in the 7 day period preceding the onset of menstruation.

It appeared to the writers that these findings, together with the dearth of well controlled data with regard to the effect of the menstrual cycle on the level of the various non-protein nitrogenous

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constituents of the blood, suggested the desirability of a more extended investigation of the influence of menstruation on the nitrogen metabolism in women.

Uric Acid and Non-Protein Nitrogen of Blood.

No constituent of the blood has, recently, been the subject of more discussion, either from the standpoint of analytical methods of estimation, or from that of significance in nitrogen metabolism, than has uric acid. It is, however, not the purpose of this paper to consider these general questions of uric acid metabolism except in so far as may be necessary in the attempt to interpret our data. For presentations of the present day views on some of the disputed points in connection with purine and uric acid metabolism, the reader is referred to the papers of Folin, Berglund, and Derick (5), of Harding, Allin, Eagles, and Van Wyck (6, 7), of Hunter and Eagles (8), of Benedict and his coworkers (9–11), of Rose (12), and to the very recent papers of Lennox (13). For presentation and discussion of the difficulties to be encountered in the actual determination of blood uric acid, also, the reader is referred to the list of references (14–26) in the appended bibliography.

There have, apparently, been so few well controlled observations of the effect of the menstrual cycle on the level of the various nitrogenous constituents of the blood in women that it seems superfluous to attempt a systematic review of the data which do exist. The recent review of Harding (27) should, however, be mentioned as covering the most significant observations on the effect of the activities of the female generative organs upon the levels of the various blood constituents during pregnancy.

EXPERIMENTAL.

The blood analyses herein reported were originally undertaken as part of an effort to determine if there were any significant cyclic variations in the nitrogen metabolism of women at the different phases of the menstrual cycle. Later, an attempt was made to check the observed variations in uric acid by different analytical methods, and to find a reason for the discrepancies between observed values for total non-protein nitrogen and the sum of the

values for known fractions of this group of blood constituents. Work on this last mentioned phase of the problem is still being continued, and only a preliminary report is included in this paper.

Subjects.

The subjects used for this investigation were students, normal in so far as could be determined by inspection of the records of the physical examinations by the staff of the University Infirmary. In many cases they were the same women who served as subjects for the investigation of blood sugar variations previously reported (28). In fact, many of the analyses were made with the same samples of blood used for the other investigation. Group I consisted of three girls who were on weighed diets, analyzed for nitrogen content and kept constant for each individual for at least 1 month at a time. Preliminary descriptions of these diets have already been published (28) and the detailed report of the metabolic study is to follow. Representative curves for uric acid, non-protein nitrogen, urea, and total creatinine for one of these subjects are shown in Fig. 1. Group II consisted, as before, of women subjects on ordinary college boarding house diets, whose records showed no evidences of abnormality during the periods of observation; while Group III consisted of women, originally expected to be in Group II, who, because of colds, influenza, or of some known deviations from prescribed rules of diet during the period of observation, could not be considered as representative normals.

Group IV was made up of nine men, medical students. For practical purposes, perhaps, this last group should be considered as consisting of two subjects who maintained approximately the same uniformity of diet as Group II, and seven men who were serving as subjects for class experiments in biochemistry, which required 3 day periods on diets of a rather extreme type. The variation in acidity of ash and in the amounts of protein taken was especially marked; hence the data from this subgroup is considered to represent almost the maximum variation to be produced in normal individuals by radical changes in diet. All blood samples were taken before breakfast in the morning and under the conditions described in the previous paper.

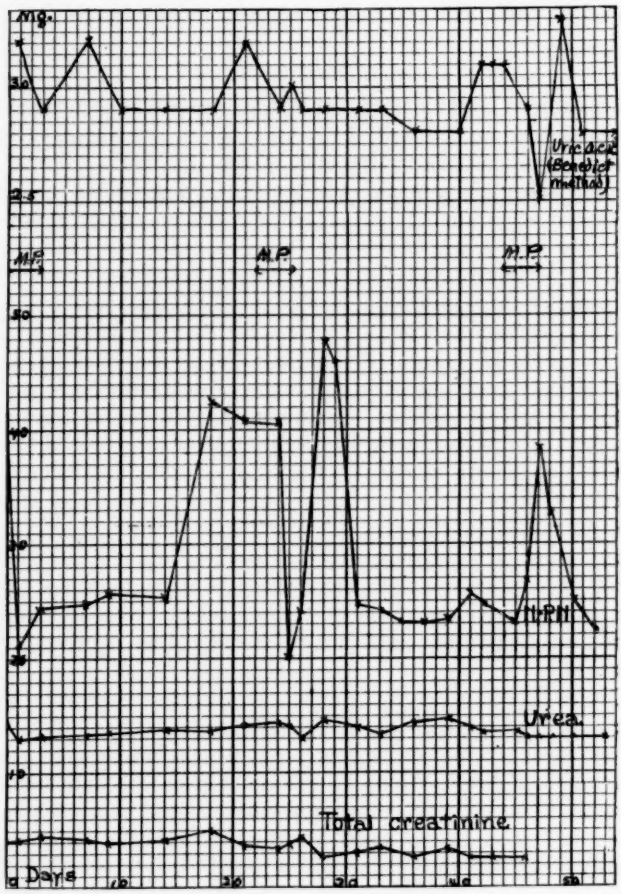

FIG. 1. Subject F. Menstrual variations in nitrogenous blood constituents in an individual on a constant, purine-low diet.

Methods.

The blood samples were taken from the arm veins, mixed with powdered lithium oxalate, and the protein precipitated according to the method of Folin and Wu (14). Care was taken to preserve uniformity of technique, especially in the addition of the 2/3 N sulfuric acid.

Analyses for uric acid by the Benedict direct method (17) were made in duplicate on all samples taken, within a few hours after precipitation of the protein. Folin's formaldehyde standard (15) was found to be more satisfactory than the original phosphate standard of Benedict, and was accordingly used. Dilute standards were prepared from the stock solution weekly, and were checked against the standards of the previous week to guard against errors due to decomposition or inaccuracies in dilution.

In perhaps half of the samples taken, analyses for uric acid were also made by the zinc precipitation method of Morris and Macleod (21). Unfortunately, the amount of filtrate available was not always great enough to justify the completion of this series.

For determinations of total non-protein nitrogen the technique of Folin and Wu (14) was followed. Determinations of blood urea, creatine, and creatinine were also made by the Folin-Wu methods (14) in a large number of samples. Since this series is not complete, and no consistent cyclic variations in these constituents of blood have been observed, these data are not included in the tables given. Most of the actual analyses for uric acid, creatine, and creatinine were made by the senior author, and those for non-protein nitrogen, urea, amino acids, and certain constituents of the "rest nitrogen" fraction by the junior author.

DISCUSSION.

Detailed reports of data for blood uric acid and non-protein nitrogen for the women in Groups I and II are given in Tables I and II respectively, while Tables III and IV represent an attempt to analyze the uric acid data from a statistical point of view. The data from the study of the women in Group III are not reported in detail, partly because of the necessity to conserve space, and partly because we were unable to designate the members of this group as

TABLE I.

Before Breakfast Blood Data, Group I: Uric Acid and Non-Protein Nitrogen.

M., age 24 yrs.				C., age 24 yrs.				F., age 31 yrs.				Remarks.
Date.	Date in relation to menstruation.*	Uric acid, Benedict method.	Non-protein nitrogen.	Date.	Date in relation to menstruation.*	Uric acid, Benedict method.	Non-protein nitrogen.	Date.	Date in relation to menstruation.*	Uric acid, Benedict method.	Non-protein nitrogen.	
1923		*mg. per 100 cc.*	*mg. per 100 cc.*	*1923*		*mg. per 100 cc.*	*mg. per 100 cc.*	*1924*		*mg. per 100 cc.*	*mg. per 100 cc.*	
Sept. 29	−4	3.6		Oct. 12	−5	3.5	29.1					Constant Diet I, mixed (contained meat). Protein, approximately 50 gm. per day.
Oct. 2	−1	2.4	28.4	" 16	−1	3.9	34.8					
" 4	+1	**2.8**		" 17	0	**2.8**	**31.2**					
" 6	+3	**2.9**	27.7	" 19	+2	**2.4**	**24.7**					
" 10	+7	3.7	28.4	" 23	+6	3.6	31.2					
" 17	−13	3.6	27.9									
" 24	−6	3.6	27.9									
				1924								
				Sept. 5	−6	2.7	25.5	Sept. 15	0		**39.3**	Constant Diet II, well balanced (meat-free). Low purine content. Protein, approximately 70 gm. per day.
				" 8	−3	2.7	23.4	" 16	+1	**3.2**	**21.9**	
				" 11	0	**2.5**	**40.8**	" 18	+3	2.9	24.3	
				" 12	+1	**2.1**	**27.2**	" 22	+7	3.2	25.8	
				" 13	+2	**2.3**	**24.3**	" 25	+10	2.9	25.5	
				" 15	+4	2.7	22.8	" 29	−7	2.9	42.2	
				" 18	+7	2.5	25.5	Oct. 3	−3	2.9	40.5	
				Sept. 22	+11	2.5	26.4	Oct. 6	0	**3.2**	**40.2**	

				Sept. 22	+11	2.5	26.4	Oct. 6	0	**3.2**	**40.2**	
				" 25	+14	2.5	32.4(?)	" 9	+3	**2.9**	**20.0**	
				" 29	+18	2.7	22.5	" 10	+4	**3.0**	**24.0**	
				Oct. 30	−5	2.7	25.8	" 11	+5	2.9		
				Nov. 3	−2	2.7	26.4	" 13	+7	2.9	48.0	
				" 5	0	**2.5**	**27.6**	" 16	+10	2.9	24.9	
				" 6	+1	**2.5**	**37.8**	" 18	+12	2.9	24.6	
				" 7	+2	**2.4**	**26.4**	" 21	−11	2.8	23.4	
				" 8	+3	2.4	24.9	" 25	−7	2.8	23.7	
				" 10	+5	2.7	26.6	" 27	−5	3.1	25.8	
								" 28	−4	3.1	24.6	
								" 29	−3	3.1	23.7	
								" 31	−1	2.9	26.4	
								Nov. 1	0	**2.5**	**39.6**	
								" 3	+3	**3.3**	**32.4**	
								" 5	+5	2.8	25.8	
								" 8	+8	2.8	22.5	
				1925				*1925*				
Nov. 1	0	**3.4**		Nov. 12	0	2.2	35.1	Jan. 18	−5	3.7	30.9	Constant Diet III. Low purine content. Protein, approximately 150 gm. per day.
" 3	+3	**3.0**		" 13	+1	**2.6**	**36.9**	" 20	−3	3.7	33.9	
" 7	+7	3.3	27.9	" 15	+3	**2.6**	**28.3**	" 23	0	**3.6**	**39.6**	
" 14	+14	3.3	27.7	" 17	+5	2.9		" 25	+2	**2 8**	**33.6**	
				" 20	+8		26.7	" 26	+3	**2.9**	**41.2**	
				" 22	+10	3.0		" 28	+5	3.4	35.1	
								" 30	+7	3.5	30.0	
								Feb. 2	+10	3.6	33.0	
								" 4	+12	3.2	32.7	
								" 6	+14	3.1	34.2	

* Numbers indicate days before (−) and days after (+) the onset of menstruation. Values for bloods taken during menstruation are printed in bold faced type.

TABLE I—*Concluded.*

M., age 24 yrs.				C., age 24 yrs.				F., age 31 yrs.				Remarks.
Date.	Date in relation to menstruation.*	Uric acid, Benedict method.	Non-protein nitrogen.	Date.	Date in relation to menstruation.*	Uric acid, Benedict method.	Non-protein nitrogen.	Date.	Date in relation to menstruation.*	Uric acid, Benedict method.	Non-protein nitrogen.	
1923		*mg. per 100 cc.*	*mg. per 100 cc.*	*1923*		*mg. per 100 cc.*	*mg. per 100 cc.*	*1924*		*mg. per 100 cc.*	*mg. per 100 cc.*	
Nov. 22	−14	3.0	27.7	Nov. 26	−14	2.6	28.2	Feb. 8	−11	3.3	31.6	Constant Diet IV. Low purine content. Protein, approximately 30 gm. per day.
" 26	−10	3.0	28.0	Dec. 3	−7	2.7	26.7	" 13	−6		33.0	
Dec. 3	−2	3.0	28.2	" 7	−2	3.0	30.3	" 18	−1	3.3	31.6	
" 5	0	**3.6**	**28.1**	" 9	0	**3.2**	**29.7**	" 19	0	**3.5**	**24.6**	
" 6	+1	**3.3**	**29.5**	" 10	+1	**2.8**	**29.1**	" 21	+2	**3.5**	**27.8**	
" 8	+3	**3.0**	**31.5**	" 12	+3	**3.1**		" 23	+4	**3.4**	**25.8**	
" 10	+5	3.4	32.7	" 14	+5	3.1	27.9	" 27	+7	3.5	29.8	
" 12	+7	3.5		" 15	+6	3.2		Mar. 3	+12	3.2	31.8	
								" 6	−7	3.0	28.3	
								" 8	−5	3.1	30.9	
								" 12	0	**3.2**	**29.3**	
								" 13	+1	**3.2**	**32.7**	
								" 15	+3	3.0	28.0	
								" 17	+5	3.1	32.4	
								" 21	+9	3.1	31.2	

normal, physically, during the period of observation. It should perhaps be stated, however, that consideration of these data does not in any way tend to alter the general indications which we believe to have been justified by the study as a whole.

While there may be some question as to the extent to which statistical treatment of the type of data summarized in Tables III and IV is justified, it does seem apparent, from inspection of these tables, as well as Tables I and II, that most women show a tendency to have a high blood uric acid level in the periods immediately preceding, and again in those just following menstruation. Moreover, some time during menstruation, there is a tendency toward a pronounced drop below the intermenstrual level for blood uric acid. Cyclic variations in the level of non-protein nitrogen are not so consistent, but we have frequently observed values much higher than the normal for the individual, at or near the time of menstruation.

Figures from the blood analyses made for the men who served as controls are summarized in Table V. The two men subjects whose dietary regimen most nearly approximated that of Group II (Xr. and Cw.), will be found to exhibit approximately the same limits of variability in blood composition as that observed for the women in this latter mentioned group during the intermenstrual periods. It should be stated that, while the changes in blood composition exhibited by the other members of this control group of men approached the magnitude of those exhibited by the women at or near the time of the menstrual period, there was absolutely no evidence of periodicity in the rise and fall in the level of the nitrogenous blood constituents in the dates of the men. The changes observed corresponded sharply to periods of extreme variation in the composition of the diets, or to those at which there was a history of infection and fever due to colds or influenza. These latter factors were observed to produce similar rises in the blood uric acid of the women in Group III.

As will be evident from inspection of Table VI, the menstrual variations in the women studied were approximately the same, whether the values obtained from analyses carried out according to the Benedict method or those from analyses carried out according to the Morris-Macleod method were taken for any given individual. The curves obtained by plotting determinations on the

TABLE II.

Before Breakfast Blood Data, Group II: Uric Acid and Non-Protein Nitrogen.

Date.	Date in relation to menstruation.*	Uric acid, Benedict method.	Non-protein nitrogen.
N., age 29 yrs.			
1923		*mg. per 100 cc.*	*mg. per 100 cc.*
Oct. 31	−2	3.3	33.6
Nov. 3	+2	**2.7**	**20.3**
" 5	+4	**2.9**	**28.8**
" 7	+6	**3.5**	**29.1**
" 16	+16	3.2	28.2
1924			
Mar. 5	−12	3.2	32.7
" 13	−4	2.8	33.9
" 18	+1	2.8	30.9
" 19	+2	**2.4**	**27.9**
" 12	+4	**2.9**	**29.4**
" 24	+7	2.8	32.4
Ij., age 20 yrs.			
1923			
Nov. 10	−12	4.0	31.2
" 20	−2	3.5	28.2
" 22	0	**3.7**	**28.5**
" 24	+2	**3.2**	
" 26	+4	4.8	33.0
Dec. 10	+19	4.0	34.8
Nd., age 31 yrs.			
1924		*mg. per 100 cc.*	*mg. per 100 cc.*
Feb. 8	−8	3.1	24.6
" 15	−1	3.1	23.7
" 16	0	**3.6**	**24.4**
" 18	+2	**2.8**	**23.8**
" 20	+4	**3.4**	**22.1**
" 27	+11	3.0	24.3
" 12		3.1	24.3
Mb., age 22 yrs.			
1924			
Feb. 11	−10	3.3	21.6
" 17	−4	3.4	30.3
" 21	0	**3.7**	**29.1**
" 25	+4	**3.2**	**29.7**
Mar. 3	+11	**3.6**	**26.7**
" 12	−5	3.0	31.5
" 17	0	3.3	29.4
" 19	+2	**3.1**	28.1
" 21	+4	**3.6**	30.6
" 24	+7	3.2	29.5
Eb., age 24 yrs.			
1923		*mg. per 100 cc.*	*mg. per 100 cc.*
Nov. 15	+2	**4.0**	
" 17	+4	**2.8**	**27.9**
" 20	+7	3.4	
Dec. 10	−2	3.9	41.4
Pt., age 23 yrs.			
1924			
Mar. 12	0	**2.2**	
" 19	+7	**2.1**	**34.8**
" 21	+9	3.1	33.0
" 24	+12	3.1	32.7
Fe., age 19 yrs.			
1924			
Sept. 1	−9	2.6	Undetermined.
" 6	−4	2.5	"
" 10	0	**3.3**	"
" 11	+1	**2.8**	"
" 13	+3	**2.4**	"
" 16	+6	2.5	"
" 23	+13	2.7	"
Pv., age 19 yrs.			
1924			
Oct. 7	−6	2.7	"
" 13	0	**2.4**	"
Pl., age 30 yrs.			

Pv., age 19 yrs.

1923			
Nov. 15	−13	3.3	28.1
" 28	0	**2.9**	**28.0**
" 30	+2	**2.0**	**29.7**
Oct. 2	+4	**2.9**	
Dec. 5	+7	3.6	33.0

Nk., age 21 yrs.

1924			
Feb. 7	+1	**3.1**	**38.0**
" 9	+3	3.1	26.0
" 11	+5	2.9	23.5
" 13	+7	3.0	22.4
" 20	+14	3.5	20.0
" 27	−8	2.9	19.5
Mar. 3	−3	2.9	22.6
" 6	0	**2.8**	**23.3**
" 8	+2	**2.8**	**27.8**

Ex., age 21 yrs.

1925			
Mar. 13	+4	3.5	32.1
" 17	+8	3.8	29.1
" 19	+10	3.5	27.1
" 24	+15	3.7	27.3
" 26	+17	3.2	24.6

Pl., age 30 yrs.

1923			
Oct. 17	−10	2.7	31.2
" 23	−4	2.8	31.2
" 26	−1	2.9	29.7
" 27	0	**3.3**	**30.9**
" 28	+1	**2.9**	**31.2**
" 29	+2	**2.3**	**31.5**
" 30	+3	**2.2**	**26.7**
" 31	+4	2.9	30.9
Nov. 7	+11	2.9	

Di., age 21 yrs.

1924			
Sept. 23	−2	3.1	
" 25	0	2.6	
" 27	+2	**2.9**	
" 29	+4	**2.9**	
Oct. 3	+8	3.0	
" 8	+13	2.9	
" 17		2.8	

Db., age 23 yrs.

1924			
Sept. 30	−10	3.2	33.9
Oct. 3	−6	3.4	31.2
" 10	+1	**3.0**	**31.5**
" 13	+4	**3.4**	**28.2**
" 17	+8	3.0	48.5
Oct. 7	−6	2.7	"
" 13	0	**2.4**	"
" 16	+3	**2.6**	"
" 21	+8	2.9	"
" 28	+15	3.0	"

Sp., age 28 yrs.

1924			
Sept. 23	−6	3.4	30.9
" 25	−4	3.0	36.6
" 29	0	**3.0**	**44.4**
" 30	+1	**3.3**	**22.4**
Oct. 1	+2	**2.8**	**29.4**
" 7	+8	3.0	38.4
" 9	+10	3.5	32.4
" 14	+15		32.7
" 17	−11	3.1	29.7
" 23	−5	3.1	30.3
" 25	−3	2.9	32.4
" 28	−1	3.2	39.0
" 29	0	**3.5**	**41.1**
" 31	+2	**2.8**	**24.9**
Nov. 3	+5	3.2	27.1
" 5	+7	2.9	26.1

Xq., age 21 yrs.

1925			
Mar. 13	−13	3.7	32.7
" 17	−9	4.0	30.9
" 19	−7	2.0	31.5
" 24	−2	4.0	29.4

* Numbers indicate days before (−) and days after (+) the onset of menstruation. Values for bloods taken during menstruation are printed in bold faced type.

TABLE II—*Concluded.*

Date.	Date in relation to menstruation.*	Uric acid, Benedict method.	Non-protein nitrogen.
Ex., age 21 yrs.—*Concluded.*			
1925		*mg. per 100 cc.*	*mg. per 100 cc.*
Mar. 31	−14	3.6	29.7
Apr. 2	−12	3.6	27.9
" 7	−7	3.5	29.4
" 9	−5	3.3	33.0
" 11	−3	3.7	30.9
" 13	0	**3.7**	**28.5**
" 14	+1	**3.3**	**28.5**
" 16	+3	**3.5**	**29.7**
Xr., age 22 yrs.			
1925			
Feb. 12	−7	3.3	Undetermined.
" 16	−3	3.2	"
" 18	−1	3.4	"
" 20	+1	**3.0**	"
" 24	+5	**3.5**	"
" 26	+7	3.9	"
Mar. 2	+11	3.5	"
" 4	+13	3.3	"
" 6	+15	3.3	"

Date.	Date in relation to menstruation.*	Uric acid, Benedict method.	Non-protein nitrogen.
Db., age 23 yrs.—*Concluded.*			
1924		*mg. per 100 cc.*	*mg. per 100 cc.*
Oct. 21	+12	3.3	31.3
" 25	−10	2.9	32.7
" 30	−5	3.0	34.5
Nov. 6	+1	**3.0**	**29.4**
" 8	+3	**3.0**	**48.0**
" 11	+6	2.9	
Ep., age 21 yrs.			
1924			
Sept. 15	0	**3.4**	Undetermined.
" 18	+3	**3.7**	"
" 22	+7	3.6	"
" 25	+10	3.6	"
" 29	+14	3.6	"
Oct. 3	+18	3.7	"
" 9	+22	3.8	"

Date.	Date in relation to menstruation.*	Uric acid, Benedict method.	Non-protein nitrogen.
Xq., age 21 yrs.—*Concluded.*			
1925		*mg. per 100 cc.*	*mg. per 100 cc.*
Mar. 26	0	4.0	31.2
" 31	+4	**3.5**	**24.5**
Apr. 1	+5	**3.6**	**32.7**
" 3	+7	4.1	33.0
" 6	+10	3.7	31.2
" 9	+13	3.6	20.9
" 13	+17	3.6	32.7
Qv., age 23 yrs.			
1924			
Feb. 7	0	**2.7**	**22.4**
" 9	+2	**2.7**	**26.6**
" 11	+4	3.4	41.2
" 13	+6	3.2	24.4
" 20	+13	2.9	32.4
Mar. 5	0	**2.4**	**36.8**
" 12	+7	3.2	29.8
Ti., age 22 yrs.			
1924			
Feb. 7	−24	3.9	31.9
" 13	−18	3.7	29.9
" 20	−11	3.8	30.9
Mar. 3	0	**4.0**	
" 5	+2	**3.6**	
" 7	+4	4.0	

TABLE III.

Distribution of Deviations from the Average Intermenstrual Values for Blood Uric Acid (Benedict Method), Groups I and II.

Expressed as Percentages of Total Cases Observed.*

Deviations, per cent†	0–2	2–5	5–7	7–10	10–15	15–20	20–25	25–30	30–35	35–40	Over 40.	Total.	Grand averages. Per cent deviation.
Average deviations, menstrual.	2.8	11.4	25.7	17.1	22.8	5.7	8.6	2.8	2.8			100	10.8
Intermenstrual...	35.3	47.0	17.6									100	2.8
Extreme deviations, menstrual.		5.4	2.7	24.3	21.6	18.9	8.1	8.1	8.1		2.7	100	15.7
Intermenstrual...	19.3	31.9	19.3	19.3	6.5	3.2						100	4.9

* Based on a total of 271 determinations covering thirty-seven monthly periods in twenty-two individuals.

† The figures in this column are based on 100 per cent as the average intermenstrual value observed for each period of each individual subject.

TABLE IV.

Distribution: Highest and Lowest Value Observed for Each Period Studied. Percentage of Total Periods Studied Falling within Given Limits of Deviation, Groups I and II.

Per cent.*	Lowest menstrual value.	Lowest intermenstrual value.	Highest premenstrual value.	Highest postmenstrual value.
Under 60.	2.6			
60–70	7.9			
70–80	10.5			
80–85	18.4			
85–90	18.4			
90–95	34.5	30.0		
95–100	5.2	66.6	6.2	14.7
100–105	2.6	3.3	43.7	47.0
105–110			28.1	23.5
110–115			15.9	11.7
115–120			3.1	
Over 120.			3.1	2.9

* 100 per cent is taken as the average intermenstrual value for each period studied. On this basis, the average of all highest premenstrual values observed is 105.8 per cent, of the lowest menstrual values, 86.4 per cent, and of the highest postmenstrual values, 105 per cent.

TABLE V.

Before Breakfast Blood Uric Acid; Benedict Direct Method.
Group IV (Men Controls).

Name.	Age.	No. of determinations.	Average value.		Highest observed value.		Lowest observed value.		Mean deviation.		Remarks.
	yrs.		*mg. per 100 cc.*	*per cent*	*mg. per 100 cc.*	*per cent*	*mg. per 100 cc.*	*per cent*	*mg. per 100 cc.*	*per cent*	
Xr.	24(?)	8	3.42	100	3.65	106.4	3.42	96.0	0.05	1.4	No special variations in diet.
Cw.	21	10	4.26	100	4.52	106.1	4.08	96.0	0.15	3.2	Little variation in diet.
Dl.	24	12	3.07	100	3.48	110.4	2.80	91.2	0.16	5.2	Dietary variations.
De.	24	10	3.17	100	3.40	107.2	2.57	81.7	0.24	7.5	Low value perhaps due to alkaline ash diet.
Hl.	22	11	3.80	100	4.12	108.4	3.42	90.0	0.20	5.2	Variable diets. Influenza.
Lz.	20	10	3.37	100	3.89	115.7	3.10	91.9	0.20	5.9	Variable diets and irregular habits.
Sq.	24	9	3.38	100	3.92	115.9	3.04	89.9	0.18	5.2	Variable diets and irregular habits.
Cl.	20	12	3.34	100	3.80	113.7	3.12	93.4	0.19	5.6	Extreme dietary changes.
Cn.	23	10	3.15	100	3.44	109.2	2.82	89.5	0.17	5.5	Extreme dietary changes.
Total.....		92									
Averages..	22.5		3.44		3.80	110.4	3.15	91.0	0.17	4.9	

TABLE VI.

Results of Parallel Analyses of Some Typical Blood Samples by the Benedict Direct and the Morris-Macleod Methods for Uric Acid.

Date in relation to menstruation.*	Uric acid, Benedict method.	Uric acid, Morris-Macleod method.	Date in relation to menstruation.*	Uric acid, Benedict method.	Uric acid, Morris-Macleod method.	Date in relation to menstruation.*	Uric acid, Benedict method.	Uric acid, Morris-Macleod method.	Date in relation to menstruation.*	Uric acid, Benedict method.	Uric acid, Morris-Macleod method.
N.			F.			Qo.			Fe.		
	mg. per 100 cc.	*mg. per 100 cc.*		*mg. per 100 cc.*	*mg. per 100 cc.*		*mg. per 100 cc.*	*mg. per 100 cc.*		*mg. per 100 cc.*	*mg. per 100 cc.*
−12	3.2	2.0	−5	3.7	2.6	0	**2.7**	**2.0**	−14	2.6	2.3
−4	2.8	2.0	−3	3.7	2.4	+2	**2.7**	**2.0**	−9	2.5	2.2
+1	**2.8**	**1.9**	0	**3.6**	**2.3**	+4	3.4	2.4	−4	3.3	2.2
+2	**2.4**	**1.8**	+2	**2.8**	**1.8**	+6	3.2	2.0	0	2.8	2.1
+4	2.9	2.0	+3	**2.9**	**1.9**	+13	2.9	2.0	+1	**2.4**	**1.5**
+7	2.8	2.0	+5	3.4	2.5	0	**2.4**	**1.4**	+3	2.5	1.7
Nk.			+7	3.5	2.5	+7	**3.2**	**1.9**	+6	2.7	2.0
			+10	3.6	2.5	Sp.			+13	2.7	2.2
+1	3.1	2.1	+12	3.2	2.5				−6	2.7	2.0
+3	3.1	2.1	−13	3.1	2.0	−14	3.1	2.5	0	**2.4**	**1.7**
+5	2.9	3.3	−11	3.3	2.0	−11	3.1	2.5	+3	**2.6**	**2.0**
+7	3.0	2.4	−6		1.8	−5	3.1	2.1	+8	2.9	2.6
+14	3.5	2.6	−1	3.3	2.1	−3	2.9	2.0	+12	3.0	2.6
−8	2.9	2.1	0	**3.5**	**2.1**	−1	3.2	2.3			
−3	2.9	2.0	+2	**3.5**	**2.0**	0	3.5	2.6			
0	**2.8**	**2.0**	+4	3.5	2.2	+2	**2.8**	**2.2**			
+2	**2.8**	**2.2**	+7	3.5		+5	3.3	1.8			
						+7	3.0	2.0			

* Numbers indicate days before (−) and days after (+) the onset of menstruation. Values for bloods taken during menstruation are printed in bold faced type.

same samples of blood by the two methods were, however, not always perfectly parallel.[1] It is unfortunate, in the light of the

[1] We believe that there is a tendency to lack of parallelism between the values obtained from analyses of the same samples of blood according to the two methods which cannot be accounted for by any lack in uniformity of technique with which the two procedures have been carried out. Since recoveries of added uric acid by both methods have been well within the limits of the experimental error inherent in such methods, we are inclined to feel that a suggestion to the effect that the fraction of the uric acid-like

doubts raised by the recently published work of Hunter and Eagles, and of Benedict and his coworkers (29), that a silver lactate precipitation method was not also used. At the time the determinations were carried out, the amounts of filtrate available did not seem to justify this procedure. We can therefore only conclude that, if we are to regard the substances determined as uric acid by the Benedict direct method as consisting of a mixture of closely related compounds, there is usually a similar variation in the fraction of this mixture which is precipitated by zinc in alkaline solution, at the different phases of the menstrual cycle.

It is very probable that blood samples taken, as these were, before breakfast in the morning, and never more than once daily, by no means represent the full measure of the menstrual variations which actually occur in the blood of any given individual. It seems quite evident from the study of our data, as well as from the observation of the symptoms of the average women during menstruation, that the greatest disturbances in metabolism take place, usually, within a very few hours of the onset of menstruation, as often preceding as following the actual beginning of menstrual bleeding.[2]

Inspection of the figures obtained in this study shows, moreover, that the different women studied tended to exhibit characteristic individual differences in the intervals from the onset of menstruation at which the greatest variations in blood uric acid were observed. While some women showed the highest premenstrual

compounds in human blood filtrates precipitated by zinc in alkaline solution may vary in the same individual from time to time is justified. The amount of this variation tends to be greater in some individuals than in others, and there is apparently no phase of the menstrual cycle at which there is a greater tendency to encounter this variation than at any other. Hence further study of the reasons for this phenomenon have been considered outside the realm of our main problem. Comparative data on some representative determinations by the two methods are given in Table VI.

[2] The number of our subjects whose menstrual periods began during the early morning hours is surprisingly small. In fact, not more than 5 per cent of our 1st day menstrual samples were taken within 4 hours of the actual beginning of menstrual bleeding. It is difficult, however, to see how a series of samples taken at exactly the same intervals from the time of the onset of bleeding could be obtained without introducing extra errors due to changed intervals after the last feeding, or to too much interference with the ordinary daily routine of living.

variations several days before the beginning of the flow, in others the highest level was not reached until a day or two after the beginning of the period. The fall from the high premenstrual uric acid level to the low menstrual level was often very rapid. In some individuals we have good reason to believe that this fall and the subsequent rise took place within 24 hours, while in others it was a matter of several days duration.

In some cases where a subject was studied over a period of more than 1 month, an extreme variation was observed during one monthly period, and a much smaller one during another. Careful checking up showed, almost invariably, a considerable difference in the actual number of hours from the onset of a menstrual period at which the samples were obtained. We are inclined to believe that if it were possible to secure complete data for any given woman over a sufficiently long period of time, it would be found that the peaks in the blood uric acid curves for each month would be found at similar intervals from the onset of menstruation. This is quite in accord with the observations of Moore and her coworkers (30, 31) on rhythmic variations in blood pressure and muscle efficiency.

It was also quite often the case that a variability in the amount of menstrual discomfort experienced by a subject existed when more than 1 month was included in the period of observation. In general, while on a uniform diet, and especially a diet which was planned to eliminate in so far as possible both constipation and excessive intestinal gas formation, our subjects reported much less than the ordinary menstrual disturbance. It should perhaps be noted that we have been able to obtain very few blood samples at a time when menstrual cramps were actually being experienced. The frequency with which such cramps were reported in records of physical examination was such that we made no effort to exclude as abnormal subjects who stated that they usually experienced some pain of this type at the beginning of the menstrual period, provided, of course, there was no other history of abnormality. But, perhaps because of our insistence on the necessity for maintenance of a rather uniform routine of diet and exercise, most of these subjects reported greatly lessened discomfort or no distress at all during the periods in which they were under observation.

Again, it would seem from inspection of the data given in Table

I, that the largest menstrual variations in blood uric acid occur when the diet is not purine-free. In fact, the long continued use of diets of this type which were predominantly alkaline in ash reaction and contained approximately the minimum amount of protein on which it was possible to establish nitrogen equilibrium seemed practically to eliminate this menstrual variation, even in subjects who had exhibited it to a marked degree on diets which contained meat.

It will be noted that, at least after the first few days, the general level of blood uric acid seemed to be higher on the high protein purine-free diets than on the low protein diets of the same type. The amounts of carbohydrate contained in the high protein and the mixed diets were approximately the same (about 250 gm.) and they were approximately isocaloric for the same individuals, hence the mixed diet, which contained only about 50 gm. of protein per day, was somewhat higher in fat than the other. The low protein diet contained approximately 350 gm. of carbohydrate and practically the same amount of fat as the high protein diet. The low protein and the well balanced purine-free diets were, however, more alkaline as to ash reaction than the other two. Hence it would seem that there is some reason to associate increased menstrual variations in blood uric acid with purine intake, with acidity of the ash reaction of the diet, and possibly, but not certainly from these data, with the increased fat content of the diet. In corroboration of these conclusions, we have found, on questioning the subjects in Group II about their dietary regimen, that the women who showed the largest menstrual variations in blood uric acid were taking diets which tended to contain more meat, and to be more acidic as to ash reaction than those subjects who showed the smaller menstrual variations. It should, however, be noted that the men in Group IV always showed increased blood uric acid during the short experimental periods on acid ash diets.

These general findings seem to be fairly well in accord with the views of uric acid retention and excretion recently expressed by Lennox (13). The feeding of high protein diets, as Folin, Berglund, and Derick (5), and Lewis and his coworkers (32–34) have observed, does undoubtedly stimulate uric acid excretion during the first few days of a period of high nitrogen intake. But there seems to be a tendency, after equilibrium is reached.

for the level of blood uric acid to rise as the rate of excretion falls. If the fat content of the diet is kept fairly constant, this would indicate, practically, that the feeding of alkaline ash high carbohydrate diets, is more to be desired in cases of uric acid retention, than that of the high protein purine-free diets suggested by Folin and Berglund.

When we seek an explanation of menstrual variations in blood uric acid, it would seem, also, that we may do well to consider the possible reasons suggested by Lennox for increased uric acid retention in the blood during fasting; namely, that it may be due to disturbances in "(1) equilibrium of electrolytes . . . .; (2) acid-base relationships of body tissues including kidney, or blood; (3) oxidation of fat with associated utilization of glucose." We believe that our studies on glucose utilization in women at the different phases of the menstrual cycle have to some extent indicated that there is an increased demand for glucose and a stimulation of the factors which lead to the utilization of this substance immediately following the beginning of menstruation in normal women. A more rapid disappearance of glucose with a lower blood sugar level 2 hours after ingestion of a stated amount was observed during the menstrual period than at any other time. This period of rapid lowering of blood sugar level, corresponds to the time at which we have found our lowest values for blood uric acid. Again, the dates at which the blood uric acid levels were highest, *i.e.* just before and just after the menstrual periods, were marked by a lessened rate of disappearance of ingested glucose from the blood stream. This suggests an added need for glucose to insure complete combustion of fat in the period immediately preceding menstruation, and that, in the normal individual, this is followed by a period of increased capacity to metabolize sugar, and hence fat, coincident with menstruation. Moreover, we have some evidence that, in diabetic individuals, there is an increased tendency to ketosis at the time of the onset of menstruation. It does not, therefore, seem entirely illogical to suggest that the increased blood uric acid levels which we have observed in normal individuals at the time of the beginning of menstruation correspond to a period of altered or difficult fat combustion, or of a tendency to alteration of the acid-base equilibrium in the tissues toward the acid side. We wish, however, to present this

only as a preliminary working hypothesis in explanation of these menstrual alterations in metabolism, and hope, in the future, to present other types of data which will confirm or refute it.

There are, meanwhile, two other possible explanations of the menstrual variation in blood uric acid which should, at least, be mentioned. The first of these is that this variation may be due to a change in blood volume, due, in turn, perhaps, to a change in the amount of water held in combination with the tissue proteins. Many of these assume a state of increased hydration at hydrogen ion concentrations only slightly higher than those usually characteristic of blood. Hence, this would seem, at first glance, to be entirely logical, especially if we are to accept the hypothesis previously stated to the effect that there is an increased tissue acidity, in the period immediately preceding menstruation, and that this is followed by a period of increased acid excretion, with over compensation. Unless we also assume, however, that the uric acid is bound up with some substance which is not readily diffusible, it is difficult to explain why there should be characteristic menstrual increases and decreases in the blood uric acid values, while there are no accompanying cyclic changes in the levels of sugar, creatine, etc.

On the other hand, if we accept Folin and Berglund's hypothesis that the kidney acts as the chief storage organ for uric acid, we may find our explanation for menstrual changes in the level of this substance in the blood stream in the alterations in circulation of blood through the kidneys, which may accompany congestion of the generative organs. The curves for uric acid variation, as illustrated in Fig. 1, are suggestively like those for blood pressure observed by Moore and her coworkers (30, 31). But, while it is easily possible to conceive of an altered equilibrium between stored and circulating uric acid, it is somewhat more difficult to see why a greatly altered kidney circulation would so affect the blood uric acid level without producing more marked changes in the concentration of certain other soluble blood constituents.

No attempt at statistical evaluation of menstrual variations in non-protein nitrogen level has been included in the tables, partly because the writers feel that, in all probability, many high values have been missed because the blood samples were not taken sufficiently frequently, and partly because further studies of these variations are to be made.

It should be stated, however, that in approximately one-third of the cases studied, values for non-protein nitrogen more than 25 per cent higher than the intermenstrual averages for the subjects concerned were observed at or near the time of the menstrual period. In a second group of approximately the same size, the highest menstrual values observed were from 5 to 25 per cent higher than the intermenstrual averages, while in the remainder, no significant menstrual changes in the non-protein level were observed.

A finding which is interesting in the light of the possible explanations for uric acid variations is that the high values for non-protein nitrogen were sometimes found in samples of blood in which the uric acid level was low. Moreover, while our data are not yet sufficiently complete so that we feel ready to make a detailed report, we do feel justified in making a preliminary statement to the effect that we have not been able to find any significant menstrual variations in urea, amino acid nitrogen, creatine, or creatinine of blood, and that we believe that the real variable must be found in the rest nitrogen fraction. No very large variations in total non-protein nitrogen were observed in the bloods of the men studied. Hence it would seem that, in the bloods of certain women, at least, there may be an accumulation of some unknown nitrogenous compound associated with the menstrual period. What the nature of this compound is, is already under investigation in this laboratory, in the hope that it may be possible to secure a more definite clue to the nature of the menstrual changes in the nitrogen metabolism of women.

SUMMARY AND CONCLUSIONS.

Before breakfast blood uric acid levels, as determined by analyses of 271 samples covering thirty-seven monthly periods in twenty-two normal college women, are reported. Three of these women were on weighed diets, constant for at least 1 month at a time, for periods of from 3 to 5 months each. It seems fair to conclude, on the basis of these data, that in most normal women there is a tendency for the blood uric acid to reach a high value immediately preceding menstruation. This has averaged 105.8 per cent of the intermenstrual levels for the individuals studied. There is, moreover, during menstruation a tendency to a fall to a value

averaging 86.4 per cent of the intermenstrual level, followed at or shortly after the close of the period by a second rise to a value averaging 105 per cent of this intermenstrual level. The curve of variation for any given individual tends to be of very much the same type, whether it is based on the values obtained from determinations of uric acid by the Benedict direct or the Morris and Macleod zinc precipitation methods.

92 observations of before breakfast blood uric acid levels in nine men have revealed no tendency to periodicity of variation. If we except values obtained when there was extreme irregularity in diet, the degree of variation observed in men is about the same as that observed in women during the intermenstrual periods.

The effect of diet on the degree of menstrual variation in blood uric acid in the individual is discussed and certain possible mechanisms involved in this variation are suggested.

A parallel series of observations on total non-protein nitrogen of blood is reported. This has included some very high values, observed during or just after the menstrual period in women. These exceeded 125 per cent of the intermenstrual averages for the individuals in approximately one-third of the cases studied, and ranged between 105 and 125 per cent in another third. Variations in the blood of the men controls were of much smaller magnitude, even when the dietary changes were extreme.

Moreover, a preliminary series of determinations of nitrogen partition in the blood of women has revealed no consistent cyclic changes in the before breakfast levels of creatine, creatinine, urea, or amino acids. Hence, it would seem that the rest nitrogen fraction must be involved. A search for proof of the nature of the compounds in this non-protein nitrogen variation is being continued in this laboratory.

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THE QUANTITATIVE ESTIMATION OF CALCIUM, MAGNESIUM, PHOSPHATE, AND CARBONATE IN BONE.

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During the last few years we have been engaged in a study of the inorganic composition of bone. Early in our work we ran into difficulties with methods. The literature contains the description of a variety of procedures for the quantitative determination of calcium, magnesium, carbonate, and phosphate in bone. In our hands, however, these have either not given reproducible results or have been so time consuming as to be useless. We therefore have been compelled to modify existing methods for calcium, magnesium, and phosphate, and have devised a new method for carbonate.

Preparation of the Bones for Analysis.

The soft tissues were carefully dissected from the bones which were then broken into small fragments, repeatedly extracted with neutral redistilled alcohol and ether, dried in the oven at about 100°C., and ground to a fine powder.

Determination of Carbonate in Bone.

The carbonate in bone was determined as carbon dioxide gas in a large model of an apparatus described originally by Van Slyke (1) for the determination of bicarbonate in small amounts of serum. From 250 to 500 mg. of bone powder were weighed to 0.1 mg., transferred to the cup of the apparatus, and washed into the arm containing the 50 cc. chamber with as small a quantity of water as possible. The air was expelled from the apparatus and a few drops of caprylic alcohol were trapped in the instrument. About 5 cc. of 5 N hydrochloric acid were added and the instrument

sealed with mercury. A vacuum was produced by lowering the mercury level to the 50 cc. mark and the lower stop-cock was closed. The instrument was inverted about twelve times and shaken to facilitate expulsion of the gas. The lower stop-cock was then turned so that it communicated with the arm containing the gas burette. A vacuum was produced and the gas expelled into the measuring burette together with as little water as possible. After a few moments the volume of gas was read and corrected to standard conditions. The method of calculation is that given by Van Slyke and is as follows:

$$\frac{\text{Volume of gas} - [\text{volume of solution} \times \alpha_{air}]}{1 - \frac{\text{volume of solution}}{\text{volume of chamber}} \times \alpha_{CO_2}} \times \frac{\text{observed pressure}}{760} \times \frac{273}{273+t} =$$

volume of gas under standard conditions.

$\alpha_{air} = (0.0255 - 0.00033 \times t)$ = volume of air dissolved in 1 cc. of solution.
$\alpha_{CO_2} = (1.412 - 0.0225 \times t)$ = " " CO_2 " " 1 " " "

0.001965 = factor for converting volume of CO_2 to gm.

Example.—10 cc. of sodium carbonate solution yielded 7.95 cc. of gas. The volume of liquid was 19.0 cc. The volume of the chamber was in every instance 50 cc., the room temperature 27°C. Atmospheric pressure was 760 mm.

$$\frac{7.95 - 19\ (0.0255 - 0.00033 \times 27)}{1 - \frac{19}{50}\ (1.412 - 0.0225 \times 27)} \times \frac{760}{760} \times \frac{273}{300} \times 0.001965 = 0.0197 \text{ gm. } CO_2.$$

Table I gives the results of the analysis of three samples of a bicarbonate solution each containing 1.99 gm. of carbonic acid gas.

The acid extract of bone powder is expelled in small portions into the cup and from there removed with a pipette and rubber bulb and transferred, along with the water used to wash the apparatus, to a beaker, filtered through No. 42 Whatman filter paper into a 100 cc. volumetric flask, and made up to 100 cc. with water. Aliquots of this solution were used for the determination of calcium, magnesium, and inorganic phosphorus.

Determination of Calcium and Magnesium.

The studies of Richards (2) and his associates defined the conditions for the quantitative separation of calcium from magnesium

and phosphate. McCrudden (3) applied these principles in devising methods for the determination of calcium in urine. Kramer and Howland (4) emphasized the importance of an exact adjustment of the reaction of the solution for the separation of small amounts of calcium from solutions of the ash of serum, and Shohl (5) recommended neutral red as the most suitable indicator. We have preferred brom-cresol purple because the color of this indicator at the reaction which is optimal for the precipitation of calcium oxalate, free of phosphate or magnesium, is qualitatively different from that which obtains at greater or lesser degrees of acidity.

20 cc. of bone solution are transferred to a 100 cc. beaker, 10 drops of indicator solution are added, and then 20 cc. of a saturated solution of ammonium oxalate. Concentrated ammonium hydroxide is added drop by drop until the solution becomes purple.

TABLE I.

*Analysis of Known Solution of Sodium Carbonate.**

Present.	Found.
gm.	*gm.*
1.99	1.94
1.99	1.97
1.99	1.97

* Contained 4.81 gm. Na_2CO_3 in 1 liter = 1.99 gm. CO_2.

Then 5 N HCl is added until it becomes definitely yellow. The precipitate which appears as the reaction approaches neutrality partly redissolves on acidification. The mixture may then be heated on the steam bath for a few minutes, cooled, and the reaction adjusted to pH 6.2 using either dilute ammonium hydroxide (one part concentrated ammonium hydroxide to nine parts of water) or normal hydrochloric acid. At the proper reaction the solution has a dirty gray color. The sample is then allowed to stand overnight to complete the precipitation. The next day the precipitate is separated by filtration through a No. 42 Whatman paper, washed carefully about four times with 10 cc. portions of the same ammonia solution. A hole is then made in the apex of the filter cone and the precipitate washed into a clean beaker with hot normal sulfuric acid, care being taken to insure complete transference of the precipitate. The solution is heated on the

TABLE II.

*Composition of Known Solutions.**

Solution.	$CaCO_3$	Na_2HPO_4	$NH_4MgPO_4 \cdot 6 H_2O$	Calcium.	Phosphorus.
	gm.	gm.	gm.	gm.	gm.
B	4.9100	4.5337	0.5174	1.962	1.0570
C	26.0079	5.8155	0.8505	10.400	1.3772
D	7.8758	6.0366	0.4894	3.142	1.3818
E	5.9095	5.9395	0.5835	2.362	1.3716
F	6.1417	4.5306	0.9622	2.460	1.1113

* Solutions made up to 1000 cc. with N hydrochloric acid. Concentrations are expressed in gm. per liter.

TABLE III.

Analysis of Known Solutions.

Solution.	Calcium in gm. per liter.		Phosphorus in gm. per liter.	
	Present.	Found.	Present.	Found.
B	1.962	1.96	1.057	1.07
		1.98		1.05
		1.94		1.05
				1.04
				1.03
				1.04
D	3.142	3.16	1.3818	1.37
		3.12		1.37
		3.16		1.39
		3.18		1.39
		3.14		1.38
		3.14		1.37
		3.14		
E	2.362	2.42	1.3716	1.41
		2.42		1.39
		2.41		1.39
		2.38		1.39
		2.39		1.38
		2.39		1.39
				1.38
F	2.460	2.45	1.1113	1.10
		2.46		1.06
		2.46		
		2.45		
		2.41		

steam bath and while still hot is titrated to a definite pink with 0.05 N potassium permanganate. The permanganate should be standardized against 0.1 N sodium oxalate after each series of determinations. 1 cc. of 0.05 N $KMnO_4$ solution is equal to 1 mg. of calcium. This simplifies the calculation (Tables II and III). The number of cc. of permanganate used is therefore equal to the number of mg. of calcium in the sample.

Table II gives the composition of the solutions of inorganic salts containing the same components as the inorganic material of bone. Table III gives the results of the analysis of the solutions for calcium and phosphorus whose composition is given in Table II.

Magnesium Determination.

The filtrate from the calcium determination is evaporated to about 10 cc. and transferred to a 50 cc. graduated centrifuge tube which tapers to a point. 1 cc. of ammonium citrate reagent and 3 cc. of 10 per cent ammonium phosphate are added, followed by about 8 cc. of concentrated ammonium hydroxide. If separation of crystalline ammonium magnesium phosphate does not begin at once, the sides of the tube are scratched with a glass rod, and the tube is allowed to stand overnight. The next day the volume is made up to 40 cc. with water, the tube contrifuged at high speed, and all but 0.5 cc. siphoned off, care being taken not to remove any crystals that may be floating on the surface. Ammonium hydroxide (four parts of water to one part of concentrated ammonium hydroxide) is added up to 40 cc. and the tube again centrifuged; all but 0.5 cc. of fluid is separated as before and the entire washing repeated. After the second washing as much of the supernatant fluid as possible is removed without disturbing the precipitate. 3 cc. of 20 per cent trichloroacetic acid and 10 cc. of water are added and after solution of the precipitate is complete, the sample is transferred to a 100 cc. volumetric flask and the following solutions[1] are added in order: 10 cc. of molybdic acid reagent, 5 cc. of 20 per cent sodium sulfite, 5 cc. of 0.2 per cent hydroquinone solution, water to 100 cc. The standards containing 0.5 and 1.0 mg. of phosphorus respectively are prepared in the

[1] These reagents are identical with those used in the Briggs (6) modification of the Bell and Doisy inorganic phosphorus method.

same manner for comparison with the unknown. The standards are set at 20 mm.

Calculation.—Reading of the unknown : 20.0 : : mg. of P in standard: mg. of P in unknown.

$$\text{Mg. of P in unknown} \times \frac{24}{31} = \text{mg. of Mg in sample.}$$

The accuracy of this method does not compare with that of the other methods described. The proportion of Mg to calcium is about 1:40, hence a slight error in the calcium determination produces an enormous error in the magnesium. However, if the average of a series of determinations is used instead of the results of a single determination, the value obtained is a fairly close approximation to the actual concentration. Table IV contains the results of the analysis of three samples of a known solution containing 0.378 gm. of magnesium in each sample. These results are better than those one usually obtains.

TABLE IV.

Magnesium Determinations in Known Solutions Containing Also Calcium and Phosphate. (*See Table II.*)

Present.	Found.
gm.	*gm.*
0.378	0.373
0.378	0.366
0.378	0.387

Determination of Phosphate in Bone.

The common practice in separating ortho-phosphoric acid from strongly acid solutions has been to precipitate the phosphate as the ammonium phosphomolybdate compound and after effecting a complete separation from the supernantant fluid, to dissolve the precipitate in sodium hydroxide solutions and reprecipitate the phosphate as crystalline ammonium magnesium phosphate. This is then dried in air and weighed, or converted into the pyrophosphate and weighed. Mathison (7) showed that ammonium magnesium phosphate is separated into crystalline form more readily when magnesium citrate is used than when magnesium chloride forms the salt in magnesia mixture. Stolba (8) suggested that

ammonium magnesium phosphate be redissolved by means of a known excess of 0.1 N hydrochloric acid. The excess of acid is then determined by titrating with 0.1 N sodium hydroxide solution. The method used in our work is a modification of one suggested by Fiske (9) for the determination of phosphates in urine. Magnesium citrate reagent keeps calcium phosphate in solution or when present in excess effects its solution if an amorphous precipitate of calcium phosphate has already formed. By using 10 cc. of the reagent even large amounts of calcium may be retained in solution and when the solution is rendered very alkaline with an excess of concentrated ammonia a quantitative separation of the ortho-phosphate as ammonium magnesium phosphate is effected.

Exactly 15 or 20 cc. of bone solution are transferred to a 100 cc. beaker. 10 cc. of magnesium citrate reagent are added followed by 8 cc. of concentrated ammonium hydroxide. The mixture is stirred and if necessary the sides of the beaker are scratched gently to initiate precipitation. The specimen is allowed to stand overnight. The next day it is filtered,[2] washed with four 5 cc. portions of 1 to 10 ammonium hydroxide, care being taken to drain well after each washing. Filter and flask are disconnected, the filter flask washed thoroughly and again connected as before, and the precipitate washed with four 5 cc. portions of 95 per cent alcohol redistilled from alkali. Pad and precipitate are then transferred to a 50 cc. Erlenmeyer flask and the adherent precipitate is washed in with a known excess of 0.1 N hydrochloric acid. The mixture is stirred to insure complete solution of the precipitate. 2 cc. of methyl red indicator solution are added and the sample titrated to a definite orange with 0.1 N sodium hydroxide. At the end-point the color should be orange, when compared with the pink color of a solution of N/15 KH_2PO_4 containing the same concentration of indicator.

Calculation.—(Cc. of 0.1 N HCl − cc. of 0.1 N NaOH solution) × 1.552 = mg. of phosphorus in the sample.

Phosphorus may also be determined according to the method of Briggs (6) after diluting the bone solution 1:20 and using 5 and 10 cc. aliquots of the dilution corresponding approximately to 0.5 and

[2] An emulsion of No. 40 Whatman filter paper is used. The filter is the one described by Fiske (9).

1 mg. of phosphorus. The rest of the procedure is identical with that used for the determination of phosphorus in the magnesium method. Table V shows that the determination of inorganic phosphorus on the bone solutions may be done either by the modified Fiske or the modified Briggs procedure.

CONCLUSIONS.

1. Methods have been described for the quantitative determination of calcium, magnesium, inorganic phosphorus, and carbonate in 0.5 to 1 gm. of bone.

2. The accuracy of the calcium method is ± 1 per cent; that of the phosphorus method ± 2 per cent. Carbonate may be determined within a margin of error not exceeding ± 1 per cent. The accuracy of the magnesium method does not exceed ± 5 per cent. The technique has been described in detail so that the results may be duplicated with regularity.

TABLE V.

Results Obtained on Bone by Modified Briggs Method and by Modified Fiske Method.

Phosphorus in bone.	
Modified Fiske method.	Modified Briggs method.
per cent	*per cent*
10.4	10.5
10.2	10.3
9.8	9.5
11.3	11.5
11.6	11.8

Table II gives the composition of the various solutions that were prepared from time to time to determine the margin of error in the calcium and inorganic phosphorus determinations.

The results in Table III indicate the margin of error in our determinations of calcium and phosphorus.

Preparation of Reagents.

Magnesium Citrate Reagent.—Dissolve 80 gm. of citric acid in 100 cc. of hot water. Add 4 gm. of magnesium oxide, and stir until dissolved. Cool and add 100 cc. of ammonium hydroxide (density 0.90) and filter. Dilute

to 300 cc. Let stand 24 hours and filter.[3] (If the magnesium oxide contains much carbonate it should be freshly ignited.) This is essentially the reagent used by Mathison (7), by Fiske (9), and by others.

Alcohol is prepared by distillation from alkali.

Methyl Red Solution.—0.004 per cent solution in 50 per cent alcohol.

M/15 KH_2PO_4 = 9.06 gm. of anhydrous potassium acid phosphate (Sörensen) (Merck's Blue Label) are dissolved in 1 liter of water. To 15 cc. of this solution add 2 cc. of methyl red solution.

Ammonium Citrate Reagent.—Dissolve 80 gm. of citric acid in 100 cc. of hot water, cool, and add 100 cc. of ammonium hydroxide (density 0.90). Dilute to 300 cc., let stand 24 hours, and filter.

Ammonium Phosphate Reagent.—25 gm. $(NH_4)H_2PO_4$ are dissolved in 250 cc. of H_2O. 25 cc. of concentrated ammonia are added and the mixture is allowed to stand overnight. The following day it is filtered, the filtrate is boiled to remove the excess of ammonia, cooled, and made up to 250 cc.

0.1 N Sodium Oxalate Standard.—6.7 gm. of sodium oxalate (Sörensen) (Merck's Blue Label reagent) are dissolved in 1 liter of water.

Dibrom-Cresol Purple Solution.—0.1 gm. is dissolved in 3.7 cc. of N/20 NaOH solution and diluted with water to 250 cc. giving a 0.04 per cent solution (Clark (10)).

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[3] The filter used is that described by Fiske ((9), p. 288).

STUDIES UPON THE INORGANIC COMPOSITION OF BONES.

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The experiments which we report were begun with the desire to determine what similarity exists between the salts in rachitic and non-rachitic bone. Also it was our intention to determine if precipitates obtained from aqueous solutions having the same inorganic composition as serum, are similar to the salts of bone. In the beginning we encountered many difficulties with the analyses. The methods which we finally adopted are embodied in a previous paper.[1]

The usual procedure employed by others has been to ash the bones, to dissolve the ash by means of acid, to determine the percentage composition of the minerals and, by difference, the water and organic material. This, as pointed out by Wells,[2] gives a very erroneous impression regarding the type of compounds present. Unless all the organic matter has been previously removed, ashing converts organic phosphorus into phosphates and carbon into carbonates. We have avoided ashing and have been concerned not with the proportion of water to organic material but with the inorganic compounds and their relative proportion.

After carefully removing the soft tissues, the bones were ground to a fine powder and extracted repeatedly with neutral alcohol and ether. The carbonate in 250 to 500 mg. of powder was determined as CO_2. The acid extract was filtered and made up to volume. Aliquot portions of this were used for the determination of calcium, magnesium, and phosphate.

[1] Kramer, B., and Howland, J., *J. Biol. Chem.*, 1926, lxviii, 711.

[2] Wells, H. G., *The Harvey Lectures*, 1910-11, vi, 102.

First we wished to determine the type of calcium phosphate present in bone and the ratio of the phosphate to the carbonate. In order to do this it is necessary to make the assumption that all the carbonate is present in the form of calcium carbonate and the small amount of magnesium as tertiary magnesium phosphate. The ratio of residual calcium to phosphorus then serves to show the type of calcium phosphate.

Table I gives the method of calculation. The number of gm. of magnesium in 100 gm. of bone multiplied by 0.86 gives the phosphorus equivalent in tertiary magnesium phosphate. When the latter figure is subtracted from the total phosphorus the so called residual phosphorus is obtained. The number of gm. of CO_2 in 100 gm. of bone multiplied by 0.91 gives the calcium equivalent of CO_2 in calcium carbonate. The number of gm. of calcium in

TABLE I.

Method of Calculation.

Humerus (0.6793 gm. of bone).

	per cent				
CO_2	3.25	3.25 × 0.91	=	2.96 gm	Ca.
Mg	0.3	0.3 × 0.86	=	0.26 "	P.
P	9.1	9.1 − 0.26	=	8.84 "	residual P.
Ca	20.1	20.1 − 2.96	=	17.1 " "	Ca.

$$\frac{\text{17.1 gm. residual Ca}}{\text{8.84 gm. residual P}} = 1.93.$$

Ratio Ca : P in $Ca_3(PO_4)_2$ = 1.94.
" " : " " $CaHPO_4$ = 1.29.

calcium carbonate subtracted from the total calcium gives the residual calcium. This value, divided by the value for residual phosphorus, gives the ratio required. In tricalcium phosphate the ratio is 1.94; in dicalcium phosphate it is 1.29.

The method of calculating the ratio of calcium phosphate to calcium carbonate is as follows:

Calculation of Calcium Phosphate to Calcium Carbonate Ratio.

CO_2 = 3.25 per cent. $\frac{3.25}{0.44}$ = 7.4 per cent $CaCO_3$.

Residual P = 8.84 per cent. 8.84 × 5 = 44.2 per cent $Ca_3(PO_4)_2$.

$$\frac{44.2}{7.4} = 6.0.$$

On account of the difficulties of using a whole skeleton we made determinations to see if the ratio of residual calcium to phos-

phorus in different bones from the same body is essentially the same. Our determinations show that this is so (Table II). It is possible, therefore, to use any of the long bones as a standard for conditions in the whole skeleton. This means also that the factors which determine the inorganic composition of the bones are operative throughout the whole body.

TABLE II.

Ratio Ca : P in Different Bones of Same Child.

Name.	Age.	Diagnosis.	Bone.	Ratio.
B.	3 days.	Normal.	Radius and ulna.	1.90
"	3 "	"	Humerus.	1.89
"	3 "	"	Femur.	1.87
"	3 "	"	Rib.	1.89
"	3 "	"	Tibia and fibula.	1.88
E.	6 mos.	Rickets.	All bones.	1.97
"	6 "	"	Humerus.	1.94
"	6 "	"	Tibia and fibula.	1.95
L.	1 day.	Normal.	Humerus.	1.92
"	1 "	"	Femur.	1.94
"	1 "	"	Tibia and fibula.	1.93

TABLE III.

Ratio Ca : P in Bones of Different Individuals.

Name.	Age.	Diagnosis.	Bone.	Ratio.
B.	3 days.	Normal.	All long bones.	1.89
L.	1 day.	"	" " "	1.93
C.	Still born.	"	" " "	1.94
X.	Adult.	?	Femur and tibia.	1.89
E.	6 mos.	Rickets.	Long bones.	1.96
By.	2 yrs.	"	" "	1.96
A.	6 mos.	"	Femur and humerus.	1.83
K.	7 "	"	Long bones.	1.96

In Table III we have chosen a few representative determinations. These show that the type of calcium-phosphorus compound present is the same in the bones of rachitic and non-rachitic children. It is also the same as that found in the bones of the newly born child or in those of the adult.

One is impressed with the constancy of the Ca:P ratio in the different bones of the same individual as well as in the bones of different individuals both rachitic and non-rachitic.[3]

This constancy of composition of the calcium : phosphorus ratio is observed in the bones of rachitic and non-rachitic rats.

[3] It is important to determine to what extent observed differences in ratio may be ascribed to error in the analyses themselves. Dr. Musselmen has been good enough to calculate this for us. We have assumed a margin of error of ± 3 per cent for each determination. This probably represents the extreme but will serve to illustrate the method of calculation. By actual analysis we have found in 100 gm. of bone:

	gm.	3 per cent error. gm.
Calcium	= 25	± 0.75
Inorganic phosphorus	= 11.6	± 0.35
CO_2	= 3.59	± 0.11
Magnesium	= 0.24	0.01

The calcium equivalent of 3.59 gm. of CO_2 = 3.27 gm. The residual calcium will, therefore, be:

$$\begin{array}{rr} 25.0 & \pm 0.75 \\ -\ 3.27 & \pm 0.10 \\ \hline 21.7 & \pm 0.85 \end{array}$$

In a similar manner the inorganic phosphorus of 100 gm. of bone = 11.6 gm. ± 0.35. The phosphorus as $Mg_3(PO_4)_2$ = 0.3 gm. ± 0.01, the error being negligible. This gives a residual phosphorus of 11.3 ± 0.36 gm. Then the variation of the Ca:P ratio due to analytic errors will equal:

$$\frac{a \pm \Delta a}{b \pm \Delta b} = \frac{a}{b} \pm \left(\frac{a \Delta b + b \Delta a}{b^2 - (\Delta b)^2} \right) \text{ approximately,}$$

where a = Ca, Δa = error in Ca, b = P, Δb = error in P.

Substituting the above values

$$\frac{21.7}{11.3} \pm \left(\frac{21.7 \times 0.36 + 11.3 \times 0.85}{(11.3)^2 - (0.36)^2} \right) = 1.92 \pm 0.14.$$

This represents an extreme condition. With an average error for each determination of only 1 per cent this becomes:

$$\frac{21.7}{11.3} \pm \left(\frac{21.7 \times 0.116 + 11.3 \times 0.28}{(11.3)^2 - (0.116)^2} \right) = 1.92 \pm 0.05.$$

These animals were chosen because it is very easy to produce active rickets in them, whereas in children it is more difficult to be sure of the activity of the rachitic process.

Ratio of Residual Ca : P in Bones of Rachitic and Non-Rachitic Rats.

Normal	1.93
"	1.92
"	1.98
"	1.91
"	1.94
Rachitic	1.98
"	1.98

Statements are to be found in the literature that there is a lower ratio of calcium phosphate to calcium carbonate in the bones of rachitic than in those of non-rachitic children. *A priori* one would expect this to be the case, for it has been shown that while the inorganic phosphorus concentration of the serum is diminished in rickets the bicarbonate suffers no reduction. Our studies show that there is this lower ratio in some instances of human rickets but not in all. This may be due to the inability to estimate accurately the activity of the pathological process. With rats this can readily be done and a distinctly lower ratio is found in the rachitic as distinguished from the non-rachitic animal.

Ratio of Calcium Phosphate to Calcium Carbonate.

Normal rat	10.8
" "	10.6
Rachitic rat	6.7
" "	6.9
Adult	7.0
Infant	7.0
"	7.9
Rachitic infant	6.0
" "	6.4
" "	5.8
" "	5.9

Preparation of Artificial Serum Solutions.

Precipitates were obtained from solutions whose calcium and inorganic phosphorus concentrations were made to correspond to

those found in the serum of rachitic and non-rachitic infants and rats. The solutions and precipitates were prepared as follows:

The artificial serum solutions were made up and precipitated in an 80 liter glazed stoneware crock. The crock was first partly filled with warm distilled water a little above incubator temperature. The proper amounts of chemically pure sodium bicarbonate and sodium chloride were dissolved in the water and a pinch of phenolsulphonephthalein added. The inorganic phosphate was then added in proper amount in the form of an analyzed solution of sodium phosphate having a pH of 7.4. 400 cc. of N hydrochloric acid were then stirred in. This changed the color of the phenolsulphonephthalein to the acid color.

In a separate flask the proper amounts of analyzed solutions of calcium and magnesium chloride were mixed and to this mixture 400 cc. more of N hydrochloric acid were added. This acid mixture of calcium and magnesium was made up to a volume of about 2 liters and slowly stirred into the large tank. By thus mixing the solutions in acid form no precipitation occurred.

The tank was then filled to the 80 liter mark with distilled water of such a temperature as to bring the entire mixture to about 40°C. The whole mixture was then stirred while a current of air was bubbled through it. As soon as the first cloud of precipitate began to appear a sample was withdrawn and the pH tested by the colorimetric method. A tightly fitting cover was then put on and sealed with adhesive plaster and the mixture allowed to stand for about 3 hours. As the precipitations were made in a warm room the temperature of the solution never varied more than a degree or two from incubator temperature.

The solution was then syphoned off and rapidly filtered through paper on a large Buchner funnel with suction. The precipitate was washed three times with distilled water, dried at room temperature, and brushed off the paper.

In making up the standard artificial serum solutions such an amount of sodium bicarbonate was added that the final concentration of bicarbonate was 0.03 N and the concentration of the sodium chloride was 0.1 N. A sufficient excess of sodium bicarbonate was added to just neutralize the hydrochloric acid added. The amount of sodium chloride thus formed was taken into consideration in bringing up the full sodium content to equal that of blood.

Precipitates obtained in this way show the same constancy of the calcium: phosphorus ratio and prove that the type of compound present is tricalcium phosphate identical with that found in human and rat bones. Table IV shows that the Ca : P ratio in the precipitates is within reasonable limits independent of the concentration of calcium or of phosphorus in the solution and

TABLE IV.
Ratio of Residual Ca : P in Precipitates.

Concentration of solution.		pH	Ratio.
Ca	P		
10.5	5.0	7.9	1.88 1.87 1.89
10.5	3.0	8.3	1.94 1.90 1.88
7.0	5.0	8.3	1.93 1.95
10.5	4.0	8.1	1.92 1.92

TABLE V.
Ratio of Calcium Phosphate to Calcium Carbonate.

Concentration of solution.		pH	Ratio.
Ca	P		
10.5	3.0	8.3	5.2
10.5	4.0	8.1	6.2
10.5	5.0	7.9	9.2
10.5	6.0	7.7	9.2

of its reaction pH, but with the calcium concentration and the reaction of the solution fixed the ratio of calcium carbonate to phosphate increases as the phosphorus concentration of the solution rises.

Furthermore when the ratio of calcium phosphate to calcium carbonate in these precipitates is determined one finds a direct relationship between the ratio and the inorganic phosphorus

concentration of the solution; the higher the phosphorus of the solution the higher the ratio of phosphate to carbonate in the precipitate (Table V).

The striking similarity between the quantitative composition of these precipitates and that of the inorganic deposit in bone might tempt one to argue a similarity in the mechanism of production of these deposits. A warning must be sounded against such an assumption since in a series of experiments reported in another paper calcification of bones *in vitro* was obtained in solutions in which precipitation did not occur.

CONCLUSION.

The calcium phosphate compound in all bones, rachitic and non-rachitic, granting the assumptions stated is tricalcium phosphate.

Different bones of the same individual have the same inorganic composition. Whenever it is possible to determine accurately the activity of the rachitic process as in rats, it can be shown that the ratio of calcium phosphate to calcium carbonate is greater in normal than in the rachitic animal. Some of our analyses show that this also may obtain in human rickets.

Precipitates made from solutions comparable in their inorganic composition to the serum of normal and rachitic animals contain essentially the same calcium phosphate compound as bone. The ratio of calcium phosphate to calcium carbonate, other conditions being the same, varies directly with the inorganic phosphorus content of the solution. This observation deserves emphasis. The fact that precipitates from artificial sera contain the same calcium-phosphorus compound as bone must not be taken to indicate that we believe calcification to be a process of simple precipitation. Elsewhere it has been shown that other factors must be taken into account.

When calcification occurs the type of calcium phosphate compound laid down appears to be independent of the inorganic phosphorus concentration of the serum. The ratio of calcium phosphate to calcium carbonate, however, is higher, the greater the inorganic phosphorus concentration of the serum.

Local conditions may determine the localization of the calcification but the composition of the deposit depends upon that of the body fluids and ultimately upon the composition of the serum.

THE MINERAL CONTENT OF HUMAN, DOG, AND RABBIT SKIN.

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Various aspects of the organic chemistry of the skin have received a great deal of attention, and the literature of this phase of the subject[1] is steadily increasing, due particularly to the realization by leather manufacturers that a fundamental knowledge of the skin is necessary to further progress in that industry.

Though the mineral constituents of the skin are small in amount, nevertheless we cannot agree with Seymour-Jones (1) that they are negligible, particularly as influencing the biochemical activity of the skin. The presence and fixed proportions of these mineral substances in the blood stream, though in comparatively minute amounts, are essential to normal metabolic processes and it has been shown (2, 3) that the basic content of the skin is intimately connected with certain of its functions.

It was in connection with the effort to elucidate further the latter problem that the paucity of the literature on the mineral content of the skin was realized. Luithlen (2) apparently published the first results on the content of bases of rabbit skin. He gave figures for the calcium, magnesium, sodium, and potassium of a few rabbits, one normal and several under the influence of various experimentally induced conditions. The investigation of Klose (4) furnished considerable data on the ash, fat, water, sodium, potassium, and chlorine content of the skin of normal infants and those with nutritional disturbances. And the recent work of McLaughlin and Theis (5) on the mineral content of the skin of the steer, cow, calf, bull, heifer, and goat comprises, ap-

[1] For literature see Seymour-Jones (1).

parently, all the data in the literature concerning this phase of the subject.

The need of further data on the content of bases in the skin of the common laboratory animals, the rabbit and the dog, and a comparison of these figures with those of man, in connection with the investigations of Klauder and Brown (6) on eczema, was evident, and it is with this purpose in view that the following results are presented.

Procedure.

The rabbits used were full grown, healthy, of both sexes, and of variable color and weight. They had been receiving the standard animal house diet of kale, beet pulp, oats, hay, and corn for several months before being used in this work. The dogs were young animals that had been fed on hospital scraps.

In order that as little blood as possible should be permitted to remain in the skin to complicate the results, the rabbits were killed by a blow on the head and bled immediately and as completely as possible. The dogs were placed under deep ether anesthesia and similarly bled. The skin, in either case, was stripped from the animal immediately after death and the hair and underlying tissue and fat removed by scraping with a knife. Removal of hair from the dog's skin was facilitated by preliminary singeing. The entire rabbit skin was used in the analysis, and a piece of approximately equivalent size was taken from the dogs. The human skins were obtained at necropsy[2] with one exception and consisted of a strip 3 to 5 cm. wide, taken from the ventral region between the clavicles and the symphysis pubis. In the one exception, Case 6596, the specimen represented a portion of the skin of an amputated leg.

In each case, after the hair and superficial fat were removed, the skin was dried to constant weight at 105–106°C. This usually required about 12 hours. The dried skin (the weight of the dried sample varied between 8 and 20 gm.), broken into small pieces, was then accurately weighed and ashed according to the method of Stolte (7). A white or nearly white ash (iron oxide) was

[2] These specimens were obtained through the kind cooperation of Dr. Eli R. Saleeby.

generally obtained in about 8 hours. Occasionally, at the end of this time, the ash still contained some carbon particles. In this event the residue was cooled, leached with distilled water containing two drops of concentrated hydrochloric acid, filtered, washed, and the undissolved portion of the ash together with the filter paper returned to the crucible and again ignited. The second ignition always gave a residue free of carbon particles. This residue was washed, with the aid of 2 cc. of concentrated hydrochloric acid, into the beaker containing the previous filtrate, which was then covered with a watch crystal and boiled on a hot plate for 30 minutes. The insoluble matter (silica) was then filtered off and washed, the filtrate being received in a 200 cc. volumetric flask. When cool the volume was made up to the mark. This solution, for convenience, will be referred to as Solution A.

Calcium.—Ammonium hydroxide was added to 60 cc. of Solution A, until the precipitate formed remained on stirring, and then sufficient hydrochloric acid just to dissolve the precipitate. The solution was warmed on the hot plate to about 50°C., about 1 gm. of ammonium acetate and 2 cc. of glacial acetic acid added, and the digestion continued on the hot plate until the mixed phosphates of iron and aluminum had settled, when they were filtered off and washed. The filtrate was concentrated to about 25 cc., heated to boiling, and 15 cc. of a boiling 4 per cent solution of ammonium oxalate added. After 2 hours at room temperature the contents of the beaker was transferred to a conical 50 cc. centrifuge tube, the beaker being washed several times with small portions of distilled water. Clark's (8) procedure for blood calcium was then followed, reserving the supernatant fluid and wash water for the magnesium determination.

Magnesium.—The combined supernatant and wash liquid from the calcium determination was concentrated on the hot plate to about 50 cc., ammonia added to faint alkalinity (phenolphthalein), and the magnesium precipitated by adding an excess of ammonium phosphate followed by sufficient ammonia to make one-tenth the final volume. After standing overnight the precipitate was filtered, ignited, and weighed as magnesium pyrophosphate.

Potassium.—20 cc. of Solution A were concentrated on the hot

plate to about 3 cc. and cooled. A bit of litmus paper was then placed in the solution and 40 per cent sodium hydroxide added drop by drop from a 1 cc. pipette while continually rotating the beaker until an alkaline reaction was obtained. The volume of alkali used was noted and the amount of potassium contained in this volume, as previously determined on a sample of this lot of sodium hydroxide, was subsequently subtracted from the total potassium found, to give the true potassium content of the skin. The alkaline mixture was then treated dropwise with glacial acetic acid until the litmus paper turned red, one drop added in excess, filtered through a small filter paper into a 50 cc. conical, graduated centrifuge tube and washed. The amounts of wash water were so adjusted that the volume of filtrate did not exceed 20 cc. 10 cc. of cobalti-nitrite reagent as prepared by Kramer and Tisdall (9) were then added and 1 hour allowed for precipitation, after which the precipitate was thrown down in the centrifuge tube and washed. In washing the precautions outlined by Kramer and Tisdall were followed and the potassium determined by dissolving the yellow precipitate in an excess of 0.01 N potassium permanganate (about 75 to 150 cc. were required) and titrating back with 0.01 N sodium oxalate. This procedure was used instead of the chloroplatinate precipitation because it was simpler, cheaper, and the potassium in potassium chloride solutions could be quite accurately determined. Recovery experiments were as a rule about 5 per cent too high.

Sodium.—60 cc. of Solution A were heated to boiling and a slight excess of barium chloride added. A bit of litmus paper was added and then solid barium hydroxide with constant stirring until the reaction became alkaline. The mixture was cooled, filtered, and washed. To the filtrate a little ammonium hydroxide was added, then an excess of freshly prepared saturated ammonium carbonate solution and the precipitate filtered and washed. The filtrate was evaporated to dryness and carefully ignited until all volatile matter was driven off. The residue was dissolved in about 5 cc. of water, a little ammonium carbonate solution added, and again filtered and washed, the filtrate being caught in a weighed porcelain crucible, in which it was evaporated, ignited below redness, cooled, and weighed. From the weight of the mixed chlorides was subtracted the weight of the potassium

previously obtained (calculated to chloride), giving the weight of sodium chloride which was then calculated to sodium.

DISCUSSION.

In Tables I, II, and III are listed the results of the determinations on the skins of the rabbit, dog, and man. It is evident that the figures for any one group of animals are quite variable, a finding

TABLE I.

Mineral Content of Skin of Normal Rabbits.

Rabbit No.	Bases per 100 gm. of dry skin.			
	Ca	Mg	Na	K
	mg.	*mg.*	*mg.*	*mg.*
58	71	28	200	134
57	59	37	243	133
61	65	44	180	150
3	67	47	150	172
4	57	39	137	155
5	60	21	172	158
63	63	38	198	122
64	53	17	205	160
65	62	52	168	178
71	86	31	191	146
2	68	45	209	153
1	51	32	181	106
52	60	23	149	188
72	66	38	192	161
79	63	39	130	102
67	57	30	241	138
DS1	71	37	196	150
77	55	28	116	173

which corroborates the work of other investigators. Thus Buckner and Peter (10), who analyzed the entire eviscerated white rat, concluded that rats coming from a common parent stock and all under identical conditions have an individual variation in growth and mineral content in both males and females which is not overcome when results are compiled from the average of three of each. Sherman and MacLeod (11) apparently did not agree with this conclusion; however, their own work with rats gave results that were quite variable. Hubner and Rona (12) found large

variations in the calcium content of the organs of cats as did Denis and Corley (13) on the organs of rabbits.

TABLE II.

Mineral Content of Skin of Dogs.

Dog No.	Bases per 100 gm. of dry skin.			
	Ca	Mg	Na	K
	mg.	*mg.*	*mg.*	*mg.*
7	34	29	187	188
8	44	28	250	175
9	31	24	181	158
10	39	28	190	214
2	42	22	188	192
5	39	21	220	257
1	58	30	214	362
3	57	37	155	395
4	41	24	233	217
6	40	25	188	222

TABLE III.

Mineral Content of Human Skin.

Case No.	Age.	Sex.	Color.	Bases per 100 gm. of dry skin.				Remarks.
				Ca	Mg	Na	K	
	yrs.			*mg.*	*mg.*	*mg.*	*mg.*	
9941	82	F.	B.	48	30	306	276	Senile dementia, syphilis.
9947	70	"	W.	55	29	399	188	Subacute peritonitis, diabetes.
10093	87	"	"	59	38	333	168	Cancer of breast.
6596	25	"	"	40	35	380	204	Bone sarcoma, specimen taken from leg.
10108	65	"	"	46	24	378	202	Chronic myocarditis.
10107	70	M.	"	59	37	408	295	" "
10175	28	"	B.	38	20	298	258	Pulmonary tuberculosis.
10174	34	"	"	34	24	331	251	Cirrhosis of liver.
10215	51	"	W.	38	27	386	339	Pellagra.
10279	76	"	"	45	31	380	208	" Arterial sclerosis.

The fact that such large variations exist when the entire animal body is analyzed does not lend support to the contention of Denis and Corley that the explanation for this variation lies in the diffi-

culty of obtaining samples of tissue which are uniformly alike, for obviously this uncertainty is not encountered when the entire body is taken for analysis. It is true that the isolated tissue may contain varying amounts of lymph, blood, fat, water, etc., but in the skin these factors, under the conditions outlined above, can play but a minor rôle in inducing such differences as are noted in the tables. Other factors, we believe, must be taken into account.

The age of the animal will certainly play a part in this variability. Hammett (14) was able to demonstrate, for instance, that the calcium, magnesium, and phosphorus content of the bones of rats varies with age. It was not possible, in the work

TABLE IV.

Average of Results on Animal Skins.

No. analyzed.	Animal.		Bases per 100 gm. of dry skin.			
			Ca	Mg	Na	K
			mg.	*mg.*	*mg.*	*mg.*
10	Man.	Maximum.	59	38	408	339
		Minimum.	34	20	298	168
		Average.	46	30	360	239
10	Dog.	Maximum.	58	37	250	395
		Minimum.	31	21	155	158
		Average.	43	27	201	238
18	Rabbit.	Maximum.	86	52	243	188
		Minimum.	51	17	116	102
		Average.	74	35	181	148

on rabbits and dogs, to more than approximate the age for these animals, and such data are therefore not included in the tables. In the determinations in human skins the ages were available, and a study of Table III will give one the definite impression that the calcium, at any rate, increases with increasing age. We believe that such great individual differences in mineral content are beyond the limits of either experimental error or error in sampling, and in another publication (3) an attempt has been made to correlate the differences in mineral content with variations in the irritability of the skin.

In Table IV are collected the average figures for the three types

of skin, together with the maximum and minimum values. A consideration of this table tends to show that though variations in mineral content of the skin may be large in the same species of animal yet distinct differences may appear among different species. Thus the calcium content in the skin of the rabbit is highest, in man next, and in the dog slightly less than in man. The magnesium figures change in the same direction. On the other hand, the figures for sodium and potassium are reversed, the skin of man having the greatest amount of these elements and rabbit skin the least, with the dog skin holding the intermediate position. An attempt to explain the significance of such differences does not come within the scope of the present paper, but one might point out that the normal diet of these animals would lead one to expect such results.

SUMMARY.

Data are presented giving the results of determination of Ca, Mg, Na, and K, in the skin of man, the dog, and the rabbit.

Individual variability in mineral content in the same species as well as distinct differences among different species are pointed out.

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LACTONE FORMATION FROM GLUCONIC ACIDS AND THE STRUCTURE OF GLUCOSE.

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INTRODUCTION.

In a previous paper[1] it was shown that when gluconic (*A*), galactonic (*B*), glucoheptonic (*C*), and mannonic (*D*) acids are allowed to stand in aqueous solution, each forms two types of lactones. The lactone with a 6-membered ring forms rapidly but to a small extent, while the "5-ring" lactone forms slowly and to a larger extent. Each acid gives a curve for rate of lactone formation which is the resultant of the two reactions. The lactone formation was observed polarimetrically.

Furthermore, 4-methyl glucoheptonic acid (*E*) and 2,3,4,6-tetramethyl mannonic acid (*F*) form only the 5-ring lactones, while 2,3,5,6-tetramethyl mannonic acid (*G*) forms only the 5-ring lactone, as predicted from chemical evidence for the structure of these substances. This method was shown to be useful in disclosing the ring structures of methylated sugars, particularly when the corresponding acids permit the formation of only one lactone.

[1] Levene, P. A., and Simms, H. S., *J. Biol. Chem.*, 1925, lxv, 31. In that article the following corrections should be made:

The letter "*l*" is omitted from equation (5), footnote 10.

Equations (6) and (8) should read:

$$K = f\mathrm{H}\frac{\alpha}{1-\alpha} \qquad (6)$$

$$-\log f = 0.30\sqrt{\Sigma i\nu^2} \qquad (8)$$

"where $\Sigma\, i\nu^2$ is the sum of all the ion concentrations i, each multiplied by the square of its valence ν. In this experiment $0.30\sqrt{\Sigma i\nu^2}$" etc.

Subsequent to the publication of the above paper, Charlton, Haworth, and Peat have applied the same principle[2] to the tetramethyl derivatives of glucose and galactose and the trimethyl derivatives of arabinose and xylose. They found that the "normal" derivatives obtained by methylation of the glucosides give 6-ring lactones; while the derivatives obtained by methylating the lactones of the 2,3,6-trimethyl acids give 5-ring lactones. The latter observation is to be expected, while in the case of gluconic acid the former observation is contrary to the prevailing conception of the structure of glucose. Glucose has been supposed to contain a 5-membered ring, whereas they interpreted these observations to indicate a 6-membered ring for glucose.

DISCUSSION.

In this paper we will give data on both the rates of formation and of splitting of the lactones of several derivatives of gluconic acid. Our observations are as follows (see Tables I and II and Figs. 1 to 4):

1. Both gluconic acid (*L*) and 2,3-dimethyl gluconic acid (*M*) form 6-ring and 5-ring lactones simultaneously (as would be expected).

2. The 3,5,6-trimethyl gluconic acid (*N*), obtained by methylation of diacetone glucose, gives *only* a 5-ring lactone.

3. The trimethyl (*O*) and tetramethyl (*P*) derivatives, obtained by methylating the methyl glucoside, give 6-ring lactones. We must conclude that the methyl groups are in positions 2,3,4 and 2,3,4,6 respectively (rather than 2,3,5 and 2,3,5,6 as previously supposed).

4. From 2 it follows that both mono- and diacetone glucose have 5-membered rings;[3] whereas (from 3) the tri- and tetramethyl glucoses derived from methyl glucoside possess 6-membered rings,

[2] Charlton, W., Haworth W. N., and Peat, S., *J. Chem. Soc.*, 1926, cxxviii, 89. They measured rates of splitting rather than rates of formation. Both methods lead to the same conclusions when only one lactone can form, but the rate of formation is of more significance when there is a possibility of two or more types.

[3] This serves as proof that diacetone glucose has the structure 2, 3–5, 6-diacetone glucose.

TABLE I.

Approximate Initial Reaction Rates in Formation (k_f) and Splitting (k_s) of Lactones in Aqueous Solutions.

A. Data from Lactone Formation.

Symbol.	Acid.	6-ring.				5-ring.			
		$t_{0.5}$	100A	100k_f	100k_s	$t_{0.5}$	100A	100k_f	100k_s
(A)	Gulonic.*	1.5	25	11.5	35	38	80	1.4	0.36
(E)	4-Methyl glucoheptonic.	2.5	21	5.8	22				
(F)	2,3,4,6-tetramethyl mannonic.	6.0	30	3.4	8				
(G)	2,3,5,6-tetramethyl mannonic.					117	75	0.44	0.15
Average.....		3.3	25	7	22		78	0.9	0.25
(L)	Gluconic.	0.40	10	17	156	(16.5)	$100\frac{26-10}{100-20}$	0.8	(3.4)
(M)	2,3-dimethyl gluconic.	0.33	15	30	165	71	$100\frac{51-15}{100-30}$	0.5	0.5
(N)	3,5,6-trimethyl gluconic.					50	44	0.6	0.8
(O)	2,3,4-trimethyl gluconic.	0.38	12	22	160				
(P)	2,3,4,6-tetramethyl gluconic.	0.35	7.5	15	185				
Average.....		0.36	11	21	167	60	48	0.6	0.7

B. Data from Lactone Hydrolysis.

Symbol.	6-ring.				5-ring.			
	$t_{0.5}$	100A	100k_s	100k_f	$t_{0.5}$	100A	100k_s	100k_f
(M)	1.27	$\frac{15}{(0.19)}$	43	11	(87)	$100\frac{49-15}{100-30}$	0.4	0.4
(N)					130	56	0.3	0.2
(O)	1.33	78	41	12				
(P)	1.2	91.5	53	5				
Average.....	1.3	85	46	9	108	53	0.35	0.3

* Galactonic (B), glucoheptonic (C), and mannonic (D) acids have essentially the same rates as gluconic acid (A).

$t_{0.5}$ = time to reach one-half equilibrium $\left(\frac{A}{2}\right)$.

A = fraction changed at equilibrium (100A = per cent changed).

which would indicate that the common form of methyl glucoside also has a 6-membered ring.

The question of the ring structure of the common form of glucose cannot, therefore, be decided solely from the structure of a derivative of glucose. Other data and observations are required to determine the ring structure of free sugars. This will be discussed in a future paper.

There are certain anomalies in the data which require consideration.

1. The reactions in these acids have a higher velocity than in acids (*A*) to (*G*) (see Table I, A). In the formation of the 6-ring lactone k_f is 3 times as large, and k_s is 7.6 times as large.

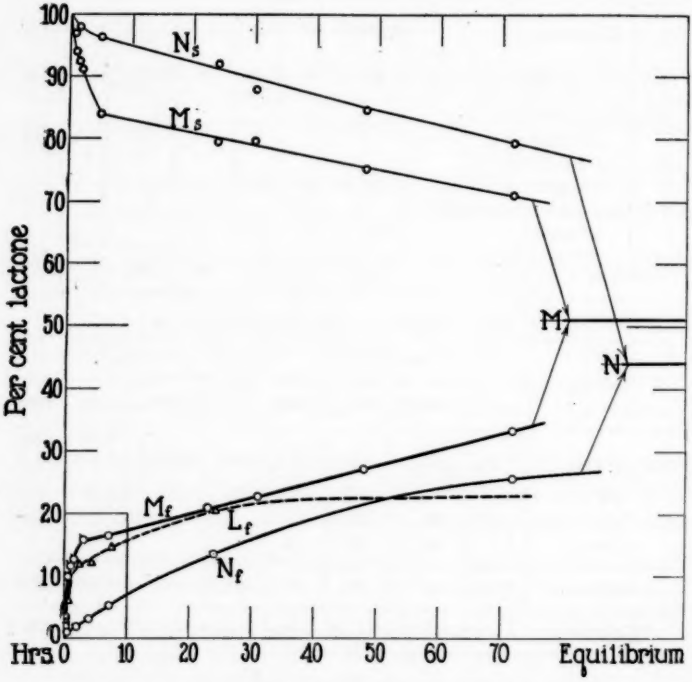

FIG. 1. Lactone formation (*f*) of acids (*L*), (*M*), and (*N*); and splitting (*s*) of lactones (*M*) and (*N*).

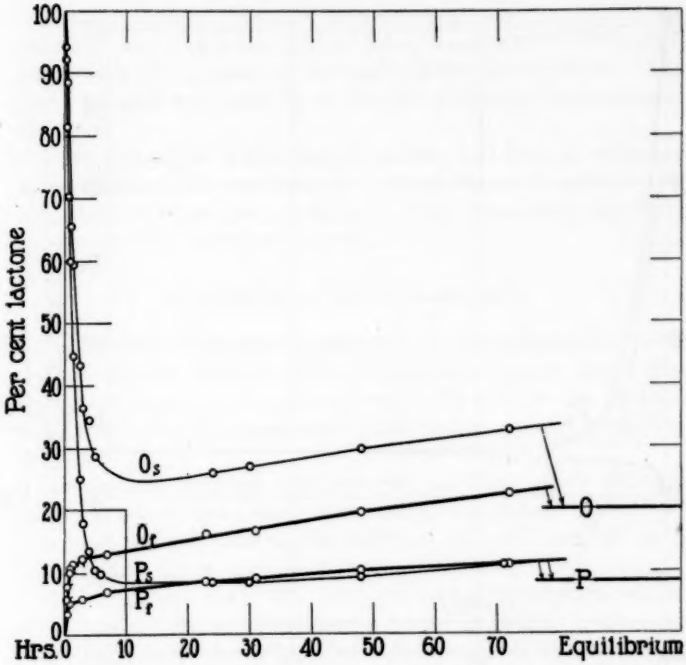

FIG. 2. Lactone formation (f) and splitting (s) of (O) and (P).

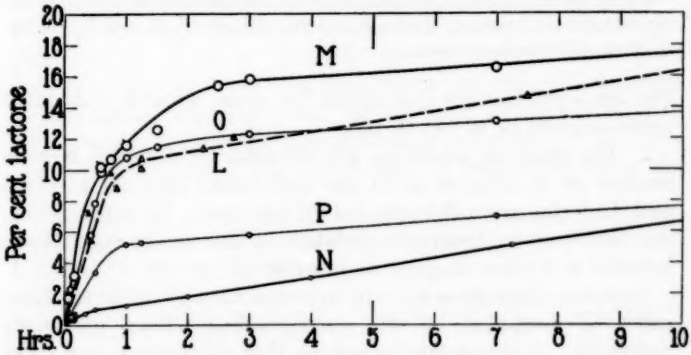

FIG. 3. Lactone formation of (L), (M), (N), (O), and (P) in the first 10 hours (see lower left-hand corner of Figs. 1 and 2).

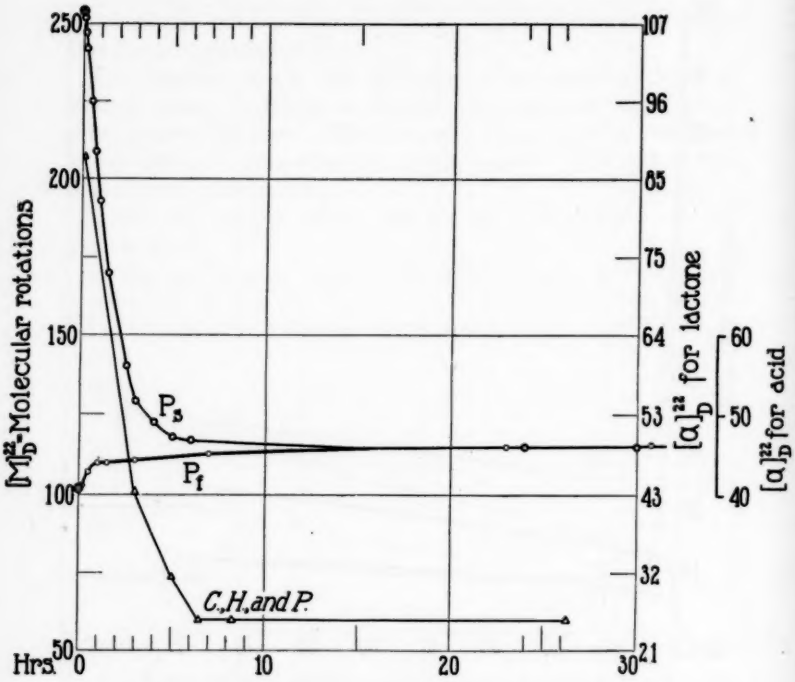

FIG. 4. Rotations in the lactone formation and splitting of (*P*) compared with the data of Charlton, Haworth, and Peat (Curve C., H., and P.) for the splitting of the same substance.

For the 5-ring lactone k_f is about the same, while k_s is about 3 times as great as in (*A*) to (*G*).

2. The resulting equilibria are different. The 6-ring lactone reaches an equilibrium of 11 per cent rather than 25 per cent and the 5-ring gives 48 instead of 78 per cent. In other words, the tendency to hydrolyze (relative to the tendency to form lactone) is 3 times as great as in acids (*A*) to (*G*).

However, these rates are only approximate and are in the same order of magnitude as the corresponding values previously measured. It seems safe to assume that the lactones are those indicated in Table I (*i.e.*, 6-membered and 5-membered respectively).

The only known difference between these experiments and the previous ones lies in the slightly lower temperature (22°C. compared with 25°C.) which would tend to lower the equilibria. The faster reaction rate must be attributed to chemical differences in the acids.

3. The δ-lactone of Charlton, Haworth, and Peat is supposed to be identical with our acid (*P*). From Fig. 4 it will be seen that their rotations are considerably lower than ours. It would appear that their material was less pure.

EXPERIMENTAL AND MATHEMATICAL.

The lactone formation was observed by optical rotation. The method was the same as previously described, except that 2 dm. open polariscope tubes (holding 4 cc.) were used for the lactone formation. The solutions were 0.250 molar aqueous solutions, as before.

The lactone splitting was observed in 0.125 molar aqueous solutions which were first placed in 4 dm. tubes. Owing to cloudiness which developed, the solutions (*M*), (*N*), and (*O*) were filtered into 1 dm. tubes and (*P*) into a 2 dm. tube.

The substances (*M*), (*N*), (*O*), and (*P*) were lactones which were dissolved directly in water for studying lactone hydrolysis. These lactones were hydrolyzed with NaOH which was then neutralized with HCl for studying lactone formation.

The results are plotted in the figures and summarized in Table I. The per cent lactone is calculated from the rotations of the pure lactones given in Table II. Except for (*L*), these were obtained by extrapolation of our curves (drawn on large scale) to zero time and agree well with the values of Levene and Meyer.[4] The lactone (*M*) is not a pure substance but a mixture of 5-ring and 6-ring lactones. The values for (*L*) were taken from the

[4] Levene, P. A., and Meyer, G. M., *J. Biol. Chem.*, 1925, lxv, 535. In that article the rotation of the free 2,3,5-trimethyl gluconic acid (now known to be 2,3,4) on p. 542, should read:

$$[\alpha]_D^0 = \frac{+0.75^\circ \times 100}{2 \times 0.976} = +38.4^\circ \qquad [\alpha]_D^0 = \frac{+0.92^\circ \times 100}{2 \times 0.976} = +47.2^\circ$$

and these values should appear in Table I.

literature. The material (*L*) was the calcium salt to which 1 equivalent of HCl was added for studying lactone formation. Since a trace of lactone may have been present at the start, 15 minutes were added to the observed time.

In Table I are the velocity constants for the various reactions. These are only approximate. They are calculated on the assumption of a monomolecular reaction with uniform catalysis. If $t_{0.5}$ is the time to reach one-half equilibrium, we may write:

$$k_f = \frac{A}{t_{0.5}} \ln \frac{A}{A - X} = \frac{2.3\ A}{t_{0.5}} \log 2 = 0.69 \frac{A}{t_{0.5}} \qquad (1)$$

$$k_s = k_f \left(\frac{1}{A} - 1 \right)$$

TABLE II.

Molecular Rotations of Gluconic Acid Derivatives.

Symbol.	Position of methyl groups.	Mol. wt. of acid.	Mol. wt. of lactone.	Type of lactone rings.	Per cent lactone at equilibrium.	$[M_A]_D$ (pure acid).	$[M_L]_D$ (pure lactone).	$[M_L]_D - [M_A]_D$
(*L*)		196.1	178.1	6 and 5	25	−3°	+122°	+125°
(*M*)	2,3	224.2	206.2	6 and 5	51	+60°	+129°	+69°
(*N*)	3,5,6	238.2	220.2	5	44	−13.5°	+100°	+113.5°
(*O*)	2,3,4	238.2	220.2	6	12	+86°	+190°	+104°
(*P*)	2,3,4,6	252.2	234.2	6	8.5	+102°	+254°	+152°

For part B (Table I) k_s and k_f are interchanged in these equations. When the 6-ring lactone is present during the formation or splitting of the 5-ring lactone it is necessary to make proper correction of (*A*).

The reaction is not necessarily monomolecular and the catalysis (with H ion) is obviously not constant. However, these values give a relative idea of the reaction velocity.

Due to change in H ion activity, the experiments (Table I, A) in which the lactone is formed from free acid, should give a higher initial velocity than those (Table I, B) in which the lactone is allowed to hydrolyze. Thus the constants in Table I, A are 2 to 4 times greater than the corresponding ones in Table I, B.

TABLE III.

Lactone Formation of Free Gluconic Acids at 22°C.

Experiment 1.

All concentrations were 0.250 molar.

Acid.....	2,3-dimethyl (*M*).*		3,5,6-trimethyl (*N*).		2,3,4-trimethyl (*O*).*		2,3,4,6-tetramethyl (*P*).*	
Length of tube.	2 dm.		2 dm.		2 dm.		2 dm.	
Time.	$[M]_D$	Lactone.	$[M]_D$	Lactone.	$[M]_D$	Lactone.	$[M]_D$	Lactone.
hrs.		*per cent*		*per cent*		*per cent*		*per cent*
0 083			(−5.0°)	(7.5)	+88.4°	2.3	+102°	0
0.167	+62.2°	3.2					+103°	0.7
0.33			(−8.0°)	(4.8)				
0.43					+92.0°	5.8		
0.50			(−8.0°)	(4.8)	+94.2°	7.9	+107.1°	3.4
0.60	+66.8°	9.9						
0.67					+96.0°	9.6		
0.75	+67.4°	10.7						
1.0	+(66.8°)	(9.9)	−8.8°	4.1	+97.2°	10.8	+109.9°	5.2
1.5	+68.7°	12.6	−9.2°	3.8	+98.0°	11.5	+110.0°	5.3
3.0	+70.9°	15.8	−8.4°	4.5	+98.8°	12.3	+110.8°	5.8
7.0	+71.2°	16.5	−7.0°	5.7	+99.6°	13.1	+112.7°	7.0
23	+74.5°	21.0	−1.6°	10.5	+102.8°	16.2	+115.0°	8.6
31	+75.8°	22.9	+0.6°	12.4	+103.4°	16.7	+115.8°	9.1
48	+78.8°	27.2	+5.4°	16.7	+106.4°	19.6	+117.6°	10.3
72	+83.0°	33.3	+11.2°	21.7	+109.4°	22.5	+118.8°	11.1
120	+91.4°	45.5			+114.4°	27.3	+112 8°	13.7

* These data are plotted in the figures (circles).

The observations of lactone formation and splitting are given in Tables III to VIII.

The curves in Figs. 1 and 2 indicate that the acids (*O*) and (*P*) do not remain at the equilibrium reached in a few hours but rise slowly. This is because the solution evaporated at the rate of about 0.07 cc. per day in spite of the fact that the tubes were covered with small inverted dishes. The effect is obvious only when 1 dm. tubes were used (holding 2 cc.). It was less when 2 dm. tubes (4 cc.) were used, and was negligible with (25 cc.) 4 dm. tubes. This has been shown by repeating the lactone split-

TABLE IV.

Lactone Formation of Free Gluconic Acids at 22°C.

Experiment 2.

All concentrations were 0.250 molar.

Acid.....	(*L*)*		(*M*)*		(*N*)		(*O*)		(*P*)	
Length of tube.	1 dm.		2 dm.		1 dm.		2 dm.		2 dm.	
Time.	$[M]_D$	Lactone.	$[M]_D$	Lactone.	$[M]_D$	Lactone.	$[M]_D$	Lactone.	$[M]_D$	Lactone.
hrs.		*per cent*		*per cent*		*per cent*		*per cent*		*per cent*
0.067			+61.2°	1.7			+87.0°	1.0	+104°	1.3
0.10	+6.0°	7.2								
0.20					−8.0°	5.5	+95.6°	9.2		
0.50					−8.0°	5.5			+106.4°	2.9
0.60	+8.0°	8.8	+67.0°	10.2						
1.0	+9.6°	10.1	+68.0°	11.6	−7.4°	6.1	+96.4°	10.0	+110°	5.3
2.5	+12.0°	12.0	+70.6°	15.4	−6.5°	7.0	+97.6°	11.2	+110°	5.3
4.5			+72.0°	17.4	−5.7°	7.8	+98.0°	11.5	+111°	5.9
7.0			+72.4°	18.0	−4.1	9.4	+98.6°	12.1	+112°	6.6
24			+76.0°	23.2	+2.1°	13.8	+102.6°	16.0	+113.6°	7.6
30			+77.0°	24.7	+4.0°	15.4	+103.0°	16.3	+113.4°	7.5
48			+79.2°	27.8	+6.4°	17.5	+104.8°	18.1	+114.8°	8.4
54.5			+80.6°	29.9	+9.6°	20.3	+106.1°	19.2	+115.6°	9.0
2 per cent concentrated HCl added (values corrected for volume).										
72			+88.0°	40.6	+37°	44.5	+104.4°	17.7	+113°	7.2
96			+90.8°	44.7	+37°	44.5	+107°	20.2	+115.4°	8.8
120			+90.8°	44.7	+37°	44.5	+108°	21.2	+115.4°	8.8

* Some of the values for (*L*) and (*M*) in this table are plotted in the figures (triangles and circles with accent mark).

ting of (*M*) and (*P*) in 4 dm. tubes sealed nearly air-tight. The results (Table VII) show that (*M*) constantly approaches its equilibrium (compare with Table VI) while (*P*) remains at the equilibrium attained in a few hours (compare with Table VI).

SUMMARY.

The previously described method of studying lactone formation of sugar acids has been applied to the formation and the splitting

TABLE V.

Lactone Formation of Free Gluconic Acids at 22°C.

Experiment 3.

All concentrations were 0.250 molar.

Acid.	(*L*)*		(*N*)*	
Length of tube.	4 dm.		2 dm.	
Time.	$[M]_D$	Lactone.	$[M]_D$	Lactone.
hrs.		*per cent*		*per cent*
0.005			−13.0°	(0.4)
0.018			−13.2°	(0.3)
0.15	+3.8°	5.4	−13.2°	0.3
0.17			−13.0°	0.4
0.33			−12.74°	0.7
0.50	+9.2°	9.8	−12.35°	1.0
1.0	+10.4°	10.7	−12.09°	1.2
2	+11.3°	11.4	−11.44°	1.8
4	+12.2°	12.2	−10.14°	3.0
7.25	+15.4°	14.7	−7.67°	5.1
24	+22.9°	20.7	+1.69°	13.4
72	+25.6°	22.9	+15.6°	25.6
80	+25.5°	22.8	+16.3°	26.2
96	+25.8°	23.0	+18.3°	28.0
120	+26.2°	23.4	+20.8°	30.2
144	+25.8°	23.0		
168	+26.2°	23.4		
	1 per cent concentrated HCl added.			
9–13 days.	+27.2°	24.2		

* The data in this table are plotted in the figures.

of lactones of gluconic acid and four of its methylated derivatives (see Table I and Figs. 1 to 3).

From the rates of reaction it was found that gluconic acid (*L*) and also 2,3-dimethyl gluconic acid (*M*) give mixtures of 5-ring lactone and 6-ring lactone (as would be expected).

The trimethyl (*O*) and tetramethyl (*P*) derivatives from methylating the glucoside give 6-ring lactones in agreement with the

TABLE VI.

Hydrolysis of Gluconic Lactones at 22°C.

Experiment 1.

All concentrations were 0.125 molar. (Plotted in Figs. 1 and 2.)

Lactone.	(*M*)		(*N*)		(*O*)		(*P*)	
Length of tube.	4 dm.		2 dm.		2 dm.		2 dm.	
Time.	$[M]_D$	Lactone.	$[M]_D$	Lactone.	$[M]_D$	Lactone.	$[M]_D$	Lactone.
hrs.		*per cent*		*per cent*		*per cent*		*per cent*
0.005							+252°	98.7
0.167	+128°	98.5					+247°	95.4
0.25					+184°	94.2	+242°	92.2
0.42	+127°	97.1			+180°	90.2		
0.50			+100°*	100	+178°	88.5	+225°	81.0
0.67	+127°	97.1	+100°	100				
0.75			+98°	98.2	+167°	77.8	+209°	70.4
1.0	+125°	94.2	+96°	96.5	+154°	65.4	+193°	60.0
1.5	+124°*	92.7	+98°	98.2	+148°*	59.6	+170°	44.8
2.0	+123°	91.3			+138°	50.0		
2.5					+131°	43.3	+140°	25.0
3					+124°	36.5	+129°	17.8
4					+122°	34.6	+122°	13.5
5	+118°	84.0	+96°	96.5	+116°	28.8	+118°	10.5
6					+(121°)	(33.6)	+117°	9.9
24	+115°	79.7	+91°	92.1	+113°	26.0	+115°	8.6
30	+115°	79.7	+86.4°	88.0	+114°	27.0	+115°	8.6
48	+112°	75.4	+82.4°	84.5	+117°	29.8	+116°	9.2
72	+109°	71.1	+77.6°	79.4	+120°	32.7	+119°	11.2
120	+111°	72.5	+64.0°	68.3	+124°	36.6	+124°	14.5
144	+115°	79.7	+60.0°	65.0	+128°	40.4	+127°	16 5
	2 per cent concentrated HCl added.							
7 days.	+113°	77	+36.0°	43.6	+124°	37	+122°	13.2
8 "	+117°	83	+38.4°	45.8	+130°	42	+124°	14.5
10 "	+126°	126	+39.4°	46.4	+136°	48	+128°	17.1

* Owing to cloudiness the solution was filtered into a 1 dm. tube at this point.

TABLE VII.

Hydrolysis of Gluconic Lactones at 22°C.

Experiment 2.

The first reading was not taken until 19 hours, when the solutions were filtered and remained clear in 4 dm. tubes, nearly air-tight.

Lactone.	(*M*)		(*P*)	
Length of tube.	4 dm.		4 dm.	
Time.	$[M]_D$	Lactone.	$[M]_D$	Lactone.
hrs.		*per cent*		*per cent*
19	+116°	81	+123°	4
23.5	+114°	78	+122°	13
42	+110°	72	+122°	13
68	+106°	66	+123°	14
90	+104°	64	+123°	14
96 (4 days).	+104°	64	+122°	13
120 (5 ")	+103°	62	+122°	13
168 (7 ")	+101°	60	+122°	13

TABLE VIII.

Lactone Hydrolysis with 2 to 4 Per Cent HCl.

All concentrations 0.250 molar.

Lactone.	(*M*)		(*N*)		(*O*)		(*P*)	
Length of tube.	1 dm.		1 dm.		1 dm.		1 dm.	
Time.	$[M]_D$	Lactone.	$[M]_D$	Lactone.	$[M]_D$	Lactone.	$[M]_D$	Lactone.
days		*per cent*		*per cent*		*per cent*		*per cent*
1	+93.6°	48.7	+36.0°	43.6	+107.2°	20.4	+111.2°	7.9
3	+95.2°	51.0	+35.2°	42.9	+107.2°	20.4	+114.0°	6.1

observations of Charlton, Haworth, and Peat which lead them to postulate that glucose has a 6-membered ring.

However, the trimethyl gluconic acid (*N*), obtained by methylating diacetone glucose, gives *only* a 5-ring lactone, which shows that a derivative may have a different composition from the parent substance. Hence, the type of ring in the common form of glucose cannot yet be decided.

The reaction rates and equilibria were somewhat different from those of the acids previously studied.

A COMPARISON OF THE FOLIN-WU AND THE NEW BENEDICT METHOD FOR SUGAR IN BLOOD AND CEREBROSPINAL FLUID.

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Benedict (1) in offering his new sugar method points out the need for a new copper solution "satisfactorily sensitive for blood sugar determination, and at the same time which would not be appreciably reduced by such known non-sugar-reducing compounds as may occur in blood or urine." This new method yields "figures averaging 20 per cent lower than those by the Folin-Wu procedure." . . . . "glucose added to blood filtrates is recovered satisfactorily by the new procedure."

What these "non-sugar-reducing substances" are is not known. Benedict does not believe that creatine, creatinine, or other known non-glucose-reducing bodies occur in the blood in large enough amount to affect appreciably the Folin-Wu reagent. Folin (2), some time ago, called attention "to the highly peculiar fact that bloods from nephritics having very high urea retention give by our original method, as by all other sugar methods, abnormally high values for the blood sugar. There does not seem to be any tangible reason why such bloods should contain any more sugar than does the blood of normal individuals. There is room for the suspicion that in such bloods other materials than sugar play an important part, that similar products in smaller amounts are present in all blood, that all sugar values obtained are high and that the lowest sugar values obtained must still be regarded as maximum values."

A recent study of nephritis with nitrogen retention afforded the writers an opportunity to compare the Folin-Wu and the new

TABLE I.

Case.	Diagnosis.	Blood.					Cerebrospinal fluid.					
		Sugar.				Non-protein N.	Sugar.				Non-protein N.	Protein.
		Folin-Wu.	Benedict.	Difference.	Folin-Wu / Benedict.		Folin-Wu.	Benedict.	Difference.	Folin-Wu / Benedict.		
1	Uremia.	120	97	23	1.2	80	82	80	2	1.02	57	144
2	"	97	91	6	1.1	84	59	55	4	1.1	61	119
3	"	91	90	1	1.0	47	63	63		1.0	37	81
4	"	97	90	7	1.1	115	57	55	2	1.04	80	100
5	"	90	78	12	1.1	35	61	59	2	1.03	20	75
6	"	122	122		1.0	40	93	100	7	0.93	20	69
7	"	97	83.5	13.5	1.1	120	63	62.5	0.5	1.0	118	138
8	"	111	100	11	1.1	200	79	77	2	1.03	168	200
9	"	175	148	27	1.2	70	125	122	3	1.02	38	131
10	"	98	88	10	1.1	143	56	60	4	0.93	105	100
11	"	105	99	4	1.1	105	56	58	2	0.96	68	175
12	"	93	85	8	1.1	125	51	57	6	0.89	89	100
13	"	111	107	4	1.0	200	87	85	2	1.02	166	212
14	"	192	154	38	1.2	170	125	125		1.0	143	119
15–1	Encephalitis.	66	55	11	1.2		46	44	2	1.02	11	44
15–2	"	83	75	7	1.1		57	54	3	1.05	7.5	78
15–3	"	111	110	1	1.0		74	78	4	0.95	10	60
16–1	"	133	118	15	1.1		56	52	4	1.07	13	50
16–2	"	109	93	16	1.2		57	59	2	0.96	15	88
17–1	"	93	79	16	1.2		67	64	4	1.04	10	35
17–2	"	84	74	10	1.1		65	60	5	1.08	10	44
18–1	"	57	46	11	1.2		77	74	3	1.03	8	50
18–2	"	89	76	13	1.2		79	68	11	1.2	11	88
18–3	"	103	97	6	1.1		69	69		1.0	8	88
18–4	"	200	161	39	1.2		140	138	2	1.01	20	56
19	"	73	69	4	1.1		59	59		1.0	16	81
20–1	"	90	76	14	1.2		50	45	5	1.11	13	75
20–2	"	124	126	2	1.0		64	62	2	1.03	15	90.
21	Miliary tuberculosis.	63	54	9	1.2		44	41		1.0	11	31
22	Congenital lues.	94	94		1.0		53	62	9	0.85	10	25
23	" "						69	69		1.0	14	44
24	Idiocy.	87	81	6	1.1		65	66	1	0.98	12	31
25	Pneumococcus meningitis.	136	121	15	1.1		5	6	1	0.83	11	244

TABLE I—*Concluded.*

Case.	Diagnosis.	Blood.					Cerebrospinal fluid.					
		Sugar.				Non-protein N.	Sugar.				Non-protein N.	Protein.
		Folin-Wu.	Benedict.	Difference.	Folin-Wu Benedict.		Folin-Wu.	Benedict.	Difference.	Folin-Wu Benedict.		
26–1	Tuberculous meningitis.	107	85	22	1.3		35	31	4	1.13	13	138
26–2	Tuberculous meningitis.	250	210	40	1.2		71	65	6	1.09	21	231
27–1	Tuberculous meningitis.	164	146	18	1.1		45	35	10	1.28	16	175
27–2	Tuberculous meningitis.	94	85	9	1.1		18	18		1.0	8	169
27–3	Tuberculous meningitis.						20	20		1.0	12	150

Results are given in mg. per 100 cc.

Benedict methods, not only on blood, but on cerebrospinal fluid drawn by lumbar puncture at the same time.

Our results are shown in Table I.

Methods.

The method of Folin and Wu (3) for blood sugar was used for both blood and cerebrospinal fluid, the only modification for the latter being in the precipitation of proteins with tungstic acid. 4 parts of spinal fluid were added to 14 parts of distilled water and to this mixture 1 part of 10 per cent sodium tungstate was added, followed by 1 part of $\frac{2}{3}$ N sulfuric acid. The flask was shaken and allowed to stand 10 minutes before filtering.

This procedure, which produces a 1:5 dilution, was followed for two reasons: The sugar content of spinal fluid is often about half that of blood and the protein is only a small fraction of that found in blood and consequently requires less tungstic acid for precipitation.

The reagents used were those at hand, which were customarily prepared fresh every few months.

The same filtrates were used for the new Benedict (1) sugar method. The reagents were all freshly prepared.

Total nitrogen in spinal fluid was determined according to the procedure of San Yin Wong (4), and occasionally the work was checked with the older method of Kjeldahl. The difference between the non-protein nitrogen content and the total nitrogen, when multiplied by 6.25, was taken as the protein content.

Blood Sugar.

In thirty-six blood sugar determinations on twenty-six cases the range by the Folin-Wu method was from 57 to 250 mg. per 100 cc. and by the Benedict method from 46 to 210 mg. per 100 cc. Except for one case (No. 20-2), the Benedict method gave lower values than did the Folin-Wu method. The average difference was 12.4 mg. In five, or 13.8 per cent of the cases, there was no difference, or a difference of only 1 or 2 mg.

Where the blood sugar is high the difference by the two methods is definitely higher than average (27, 38, 40, 18 mg.). Only two cases (Nos. 1 and 25) with normal blood sugar had differences as high as 25 and 22 mg.

In those cases where the blood sugar was low the differences by the two methods were only average.

Non-Protein Nitrogen Content of Blood.

We have accurate figures for non-protein nitrogen in the first fourteen cases. Where there is no nitrogen retention we have found that the blood non-protein nitrogen is usually about double the non-protein nitrogen in the cerebrospinal fluid. In analyzing twenty-eight cases with a blood non-protein nitrogen 40 mg. or lower, the cerebrospinal fluid non-protein nitrogen was found to be an average of 46.5 per cent of the blood non-protein nitrogen. It is fair to assume then that in the remaining cases the blood non-protein nitrogen is within normal limits, since there is no increase in the cerebrospinal fluid non-protein nitrogen in these cases.

It will be seen that there is no relationship between blood non-protein nitrogen and differences in blood sugar by the two methods. In those cases with high non-protein nitrogen there was just as

much or as little difference in blood sugar by the two methods as in those cases with no nitrogen retention.

Hyperglycemia in Nephritis.

The nephritic hyperglycemia reported by other observers is present in only two of our cases. The differences in these cases were maximum (38, 27 mg.). These cases were all severe nephritics with high blood pressure, retinal changes, and a typical urinary and blood chemistry picture. Folin (5), in a recent paper, says that:

"While a part of the high sugar values obtained in nephritic blood may be represented by reducing products which are not sugar or sugar derivatives, it is not only possible, but highly probable that a very large fraction of these unknown reducing materials is of exactly the same sort as the non-glucose carbohydrate materials which occur in normal urines. These more or less foreign and unusable sugars and sugar derivatives which are eliminated by normal kidneys would naturally accumulate in the blood when the kidneys fail to work."

Since not all severe nephritics show this hyperglycemia (in our series only 14.3 per cent) some other explanation must be looked for. In certain other conditions a high blood sugar found by various methods has been reported occasionally. These conditions, convulsions due to different causes, epidemic encephalitis, tuberculous meningitis, are non-diabetic and, while there might be some impairment of kidney function, it is not severe enough to show the changes that go with real nephritis. A satisfactory explanation of these changes awaits further investigation.

Cerebrospinal Fluid Sugar.

In thirty-eight determinations on twenty-seven cases the range by the Folin-Wu method was from 5 to 140 mg. per 100 cc. and by the Benedict method from 6 to 138 mg. per 100 cc. Most of the values were lower by the Benedict method, but in nine cases, or 23.7 per cent, this method gave higher values than did the Folin-Wu. The average difference by the two methods was 3.1 mg., which is only one-fourth the average difference found in the blood.

In twenty, or 52.6 per cent of the cases, there was no difference,

or a difference of but 1 or 2 mg. This is in marked contrast to the findings in blood sugar where only 13.8 per cent showed practically no difference. In 21 per cent of the cases there was a difference of 5 mg. or more.

Abnormally low or high sugar values in the cerebrospinal fluid did not cause more than the average differences by the two methods. This again is in contrast to the blood findings where abnormally high sugar values gave maximum differences.

Cerebrospinal Fluid Non-Protein Nitrogen.

It will be seen at once that there is no relation between the non-protein nitrogen content of the cerebrospinal fluid and the differences in sugar content by the two methods.

Cerebrospinal Fluid Protein.

A study of the table will show the absence of any relationship between the protein content of the cerebrospinal fluid and the differences in sugar content by the two methods.

DISCUSSION.

The average difference in cerebrospinal fluid sugar by the two methods was only 3.1 mg. and more than half of the cases showed practically no difference. This would indicate that the interfering substance is not present in the cerebrospinal fluid so constantly nor in such amounts as in the blood. Yet the fact that in 21 per cent of the cases there was a difference of 5 mg. or more shows that this unknown substance can and does get into the fluid, probably through the choroid plexus. Whether this happens in a normal individual or not, we do not know. In hospital practice it is difficult to determine whether we are dealing with a normal cerebrospinal fluid and an intact central nervous system or not. It is well known that certain substances pass the choroid plexus more readily than others, and that this permeability is affected by disease and by changes in the composition of the blood. Many of our cases show a disturbance in the normal relation between the blood and the cerebrospinal fluid sugar. Normally, the sugar and the non-protein nitrogen content of the cerebrospinal fluid is approximately half that of the blood.

In thirteen of the cases the sugar in the fluid was 70 per cent or more of the blood sugar, and in one case it was 135 per cent of the blood sugar. In only two of the fourteen cases of nephritis was the normal relation of the blood and cerebrospinal fluid non-protein nitrogen maintained, and in these cases the non-protein nitrogen in the blood was normal, in one 40 mg. and 70 mg. in the other. The other cases all showed a high ratio, and one showed practically the same non-protein nitrogen content in the cerebrospinal fluid as in the blood.

SUMMARY.

1. Simultaneous blood and cerebrospinal fluid sugar determinations were made on twenty-six cases by the Folin-Wu method and by Benedict's new method.

2. In the majority of cases the Folin-Wu method gave higher values than did the Benedict method.

3. In the blood the average difference by the two methods was 12.4 mg., with 13.8 per cent of the cases showing practically no difference.

4. The non-protein nitrogen content of the blood had no relation to differences in sugar content by the two methods.

5. In the cerebrospinal fluid the average difference was 3.1 mg. with 52.6 per cent of the cases showing practically no difference.

6. The interfering substance or substances are not present in the cerebrospinal fluid so constantly or in such large amounts as in the blood.

7. The protein and non-protein nitrogen content of the cerebrospinal fluid has no relation to differences in sugar content by the two methods.

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THE ESTIMATION OF SUGAR IN BLOOD AND NORMAL URINE.

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About a year ago the writer (1) described technique for the determination of sugar in blood which yields figures appreciably lower than those obtained by the use of the older methods. Since the publication of that paper two investigators, employing widely differing procedures, have reported results which indicate the correctness of the lower figures obtained with the new method. Harned (2), after completely precipitating the nitrogenous constituents from the blood by the use of mercuric nitrate and sodium bicarbonate, obtained figures for the sugar by use of the Folin-Wu method which agree quite closely with those obtained by the Benedict procedure on the tungstic acid filtrate.

Folin (3) has proposed new reagents for the determination of sugar in blood and urine. The figures reported by the new procedure show essential agreement with those obtained by the new Benedict method. In this latest contribution to the subject of blood sugar determination Folin has subjected the new Benedict procedure to a very searching critique. Indeed Folin's paper would seem to lead to the rather anomalous conclusion that while the new Benedict method yields figures which are probably correct, the method itself is quite unsatisfactory. Obviously the main point of interest is the question as to what figures most nearly represent the true glucose content of the blood. Of much less importance is the particular technique employed to obtain such figures. It is therefore only because we feel reasonably confident that our new technique, especially in the modified form described below, is somewhat more convenient and decidedly safer for general use in biochemical determinations of sugar than is the new method described by Folin, that the following discussion is presented.

Early in his paper (3) Folin remarks, "Benedict seems to believe that the only essential feature of his qualitative sugar reagent is the fact that its alkali is a carbonate instead of a hydroxide. This concept I think is not quite correct." A little later in the same paper Folin suggests a simple experiment to prove his contention that the citrate reagent is less sensitive than is a tartrate reagent. That the present writer held no such mistaken opinion as Folin would imply is shown by the following quotation from our recent paper (1). "This [tartrate] reagent is more delicate than the citrate reagent subsequently proposed, but was not so stable nor so satisfactory, because of its great sensitiveness as a general qualitative reagent for sugar in urine." In view of the fact that we thus clearly recognized and called attention to the greater delicacy of the tartrate solutions (although, as we pointed out, the sensitiveness is gained at the cost of a loss in specificity) it is not clear why Folin should have so elaborated on this point.

It is quite likely that any marked gain in specificity for glucose by an alkaline copper solution will result in production of less cuprous oxide from a given amount of glucose under fixed conditions. This is as true of Folin's new copper solution as it is of our citrate reagent. In the case of Folin's new reagent the expedient resorted to to gain a normal yield of cuprous oxide is to increase the period of heating almost 100 per cent. We have employed sulfite to accomplish the same purpose. From the practical standpoint the sulfite offers the better solution to the problem, and we fail to find any theoretical reason against its use as we employ it.

Folin has objected on several grounds to the use of the complex tungstic acid reagent which we employ for color production. Thus he says that the acidity of the final mixture is so low that "the mixture will develop a blue color with uric acid." We suggest that anyone interested in the practical bearing of this criticism try the effect of uric acid added in quantity equal to ten or more times the amount ever present in blood filtrates, before color development. Such a simple experiment should convince anyone that there is no practical basis for the criticism. Discussing the color reagent still further Folin raises objection because the cuprous oxide dissolves very slowly and says, "Benedict . . . . intended to provide for this flaw in his method by inserting a waiting period of 10 minutes between the addition of the uric acid reagent . . . . and the dilu-

tion with water." In these days when speed is one of the prime considerations in the use of an analytical method the waiting 10 minutes for color development may be something of a drawback in the use of the method, but we are quite unwilling to admit that there is any flaw at this point. By imposing conditions which the new technique was never intended to meet, Folin showed that some cuprous oxide might remain undissolved during the 10 minute period. Such results are never obtained when the method is used as directed. It is perhaps interesting to note that both of Folin's criticisms of the color reagent should, if valid, result in high figures for the blood sugar, a defect which the method does not appear to exhibit in practical use. The actual figures yielded by the method seem to give ample vindication of our use of the complex tungstic acid reagent.

In the modified form of the method given below the use of the tungstic acid color reagent has been retained, in spite of the fact that long ago we developed, and for a time used, a molybdic acid reagent which gave a satisfactory color yield with the citrate copper reagent. Although Folin so strongly champions the complex molybdic acid reagent for color development, and has described a new reagent of this type which is prepared in quantity only with some difficulty, it is our opinion that the molybdic reagents are perhaps not so desirable as those containing complex tungstic acid. Folin states that even his new molybdic reagent is not very satisfactory for color development with our new copper reagent, because of the "huge" citrate content of the latter. Yet as a matter of fact the citrate in this copper reagent can be reduced some twenty or thirty times and still show a marked effect upon color development with the molybdic reagent. If so seemingly innocuous a substance as sodium citrate, which has neither oxidizing nor reducing properties, can seriously impair color development with the molybdate reagents, it would seem that for biochemical work in general, where unknown constituents are practically always present in one solution and not in the standard, it would be safer to adopt the tungstic acid reagent, which is not so susceptible to the effects of foreign compounds. Especially is this so since there appears to be no reason against adoption of this reagent. The latter reagent yields an intense pure blue coloration with cuprous oxide, while the molybdic reagents yield a greenish

blue shade at first, the greenish tint rapidly disappearing on standing, so that a freshly diluted solution cannot be compared in a colorimeter with one which has stood for even a relatively short time.

Folin has called attention to one serious defect in the method which we presented; *viz.*, that the sulfite content of the reagent becomes rapidly oxidized so that the mixed solution is useful for only a very limited time. This criticism is fully justified, and we much regret the error which at this point appeared in the published form of the method. There would be little gained by going into a detailed account of how this trouble arose. It will be sufficient to say that for more than 2 years prior to publication the reagent had been prepared at intervals and as so prepared contained nearly 2 per cent of "sodium bisulfite" as contained in a bottle of Kahlbaum's product which had been in the laboratory for some years. After the paper was in the form of page proof we discovered that fresh sodium bisulfite could not be used in nearly the quantity indicated in the older formula. The only opportunity for change in the paper then was to alter the quantity of bisulfite to that which we knew could be added without detrimental results. Subsequent work has shown that sodium bisulfite is not a wholly definite product, some samples containing a relatively large proportion of meta-bisulfite, and others very little. In the modified form of the method detailed below we have therefore abandoned the use of bisulfite. Sodium sulfite is now used in an amount allowing for a considerable excess of this salt, and proper precautions are taken so that under even the most adverse conditions of keeping the reagent as used will always contain an adequate amount of sulfite.

Folin's new bicarbonate-containing copper reagent owes its specificity to decreasing the alkali content to a very minimum. Consequently this new reagent has not sufficient alkali to take care of even minute amounts of acid, nor can more than traces of alkali be added without wholly upsetting the hydrogen ion content of the solution. Folin frankly admits these facts, and yet he rather minimizes the necessity for neutralization of the blood filtrates prior to the sugar determination. According to our experience with the new reagent exact neutralization of all filtrates is quite essential if the figures obtained are to have any significance. It is

doubtful whether such a delicately balanced solution is best for general use. The lack of any reserve alkali whatever in the solution is apt to lead even very careful workers into error when the ground of operations is changed in any way. As will develop later in this paper, Folin has himself apparently fallen into serious error in assuming that his new copper reagent can be applied to the determination of sugar in normal urine along the lines of accepted procedure for this analysis.

The following technique for the determination of blood sugar is essentially the same as that previously proposed (1), with modifications which make for greater speed and convenience in the analysis, and with provision for always having present an adequate amount of sulfite. Through addition of an ammonium salt to the copper reagent the reduced copper is held in solution, so that color development is practically immediate after addition of the color reagent. We believe it to be at least a theoretical advantage to have the reduced copper in the same condition in both standard and unknown. In all other copper methods with which we are familiar the cuprous oxide is always in a more finely divided form in the blood filtrate than in the standard, and this introduces the chance of greater reoxidation in one than in the other, or of a difference in relative intensity of action upon the color reagent. The complex tungstic acid color reagent has been somewhat changed in composition. Owing to the increased quantity of sulfite in the copper reagent the formaldehyde has been increased in the color reagent. Sodium chloride has been added to increase the specific gravity of the solution so that it mixes more promptly with the other solution after reduction of the latter, and hydrochloric acid has been added to provide for higher acidity of the total mixture. A portion of the hydrochloric acid is, however, neutralized by the additional carbonate content of the copper reagent. The carbonate in the latter reagent has been somewhat increased—slightly more than enough being added to react with the ammonium salt now contained in the reagent. While our reagents yield a slight blank, the color under such conditions is so slight that we have made no effort to improve the method in this respect. Anyone who may feel that the color from a blank may be significant should try the experiment of running a blank side by side with a standard. When the two are diluted it will be

readily seen that the color of the blank is so faint as to be entirely unobjectionable, especially since it is always present in both standard and unknown.

The procedure for the method follows.

Reagents.

1. Alkaline Copper Solution.—

Pure crystallized copper sulfate	6.5 gm.
Sodium citrate	200.0 "
" carbonate (anhydrous)	60.0 "
Ammonium chloride	9.0 "
Distilled water to make	1000 cc.

Dissolve the citrate and carbonate together in about 800 cc. of water. Dissolve the copper sulfate separately in about 100 cc. of water and add to the other solution with stirring or shaking. Add the ammonium chloride, dilute to 1 liter, and mix.

Not more than 1 month before use add to each 100 cc. of the reagent 2.5 to 3.0 gm. of pure anhydrous sodium sulfite. This can be conveniently done as follows: Select a bottle holding about 100 cc., and when desired fill it from the main reagent bottle. Select a porcelain spoon or other convenient measure which will hold 2.5 to 3 gm. of sodium sulfite and use this to measure the sulfite for addition to the small reagent bottle. Even a quite wide variation (nearly a whole gm.) in the quantity of sulfite added will not appreciably affect the results with the method. Where only occasional sugar determinations are to be made it may be more convenient to add the sulfite from a 20 per cent solution kept in a dropping bottle. Where this method of addition of sulfite is used, 5 drops of the 20 per cent sulfite solution should be added to each sugar tube prior to adding the other solutions. The strong sulfite solution will keep satisfactorily for at least 4 months in well filled tightly stoppered bottles, and probably two or three times this length of time. We have not yet had opportunity to find out just how long the sulfite-containing solutions will keep. The periods given are minimal.

2. Complex Tungstic Acid Color Reagent.—This is prepared as follows: 100 gm. of pure sodium tungstate are placed in a liter flask and dissolved in about 600 cc. of water. 50 gm. of pure

arsenic pentoxide are now added, followed by 25 cc. of 85 per cent phosphoric acid and 20 cc. of concentrated hydrochloric acid. The mixture is boiled for 20 minutes. After cooling add 60 cc. of commercial formalin, 45 cc. of concentrated hydrochloric acid, and 40 gm. of sodium chloride. Dilute to 1 liter and mix.

The technique of the method is as follows: 2 cc. of the 1:10 tungstic acid filtrate are measured into a Folin-Wu sugar tube,[1] followed by 2 cc. of the copper reagent. The contents are mixed by side to side shaking for a moment, and the tubes placed in boiling water for 5 minutes. The tubes are then cooled by immersion in cold water and 2 cc. of the complex tungstic acid color reagent are added. After 1 to 2 minutes the contents of the tubes are diluted to 25 cc. with water, thoroughly mixed, and compared with a standard in a colorimeter. As standard solution, pure glucose solution containing 0.1 mg. of glucose per cc. is employed. This can conveniently be prepared by 1:10 dilution of a 0.1 per cent solution of pure glucose in water, preserved with 1 or 2 cc. of toluene. This solution will keep indefinitely so long as any toluene remains upon the surface of the solution. The diluted standards will also keep for long periods in the presence of a few drops of toluene.

[1] In our previous paper we suggested the substitution of an ordinary graduated test-tube and the use of benzene, as an alternative for the use of the Folin-Wu sugar tubes. Folin (3) thinks that such substitution cannot safely be made, and offers a fanciful, and quite incorrect explanation of our findings in this matter. Apparently we were not specific enough in our directions. There are two important questions involved. These are the width of the tube and the quantity of benzene employed. If one uses a wide tube and a minimum of benzene, reoxidation is little affected. With a moderately narrow tube (15 mm. or less, internal diameter) and enough benzene to leave even a trace on the surface of the solution at the end of the heating, reoxidation is as low as when the Folin-Wu sugar tubes are used when used with the technique we previously proposed. The common impression that there is no reoxidation when the Folin-Wu sugar tubes are employed is not correct. By comparing results with sugar tubes which have been evacuated with a good suction pump (and the vacuum maintained during the period of heating and cooling) with those not so treated, it will be found that as usually employed the Folin-Wu sugar tubes permit 6 to 7 per cent reoxidation, against more than 20 per cent in the wide straight tubes first used in the Folin-Wu method. We consider the Folin-Wu tubes a very valuable contribution to sugar analysis, and advocate their use in the procedure described in the present paper.

The degree of accuracy of the method is satisfactory where the solution analyzed is not more than twice as strong or half as weak as the standard. Results by the modified method for blood sugar are in quite close agreement with those previously reported (1).

Folin has advocated application of his new bicarbonate copper solution to the determination of normal urine sugar in Lloyd's filtrates, and reports figures where the new reagent is employed which are as low as one-half to one-third those obtained on the same filtrates with the Folin-Wu reagent. It seems, even at first glance, doubtful whether these excessively low figures reported by Folin for urines can be correct. We therefore first tried the effect of Lloyd's reagent upon the standard glucose solution, using the Lloyd's exactly as Folin and Berglund (4) employed it, and Folin's new copper reagent in place of the Folin-Wu solution. When Folin's new reagent is substituted for the Folin-Wu copper solution, treatment of the standard glucose solution with Lloyd's reagent results in an apparent loss of glucose up to 30 per cent. Apparently the loss is more or less proportional to the length of time of shaking the mixture with Lloyd's, or to the length of time it remains on the filter. Hence we infer that the amount of Lloyd's which dissolves determines the sugar loss. Doubtless the loss is due to withdrawal of hydroxyl and carbonate ions through precipitation which occurs when the filtrate is heated with the copper reagent. It is not due to the faint acidity which remains after treatment with acid and Lloyd's. The latter reagent itself neutralizes most of the added acid, and exact neutralization of the trace of acid left does not appreciably affect the results. A single experiment on recovery of sugar added to urine by the new Folin method showed a loss of about 25 per cent of the 40 mg. added. Obviously the new Folin procedure is not adapted to the determination of sugar in normal urine. This finding serves to emphasize the caution necessary in the use of the new Folin bicarbonate copper reagent.

For determination of sugar in normal urines the reagents described earlier in this paper probably yield the most nearly correct results. The method is carried out exactly as described by Folin and Berglund with the use of Lloyd's reagent, save that the new reagents are substituted for the Folin-Wu copper and molybdic acid reagents. Results are slightly lower than where the Folin-

Wu reagents are employed, but there is not a great deal of difference between figures by the two methods.

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CAN PURINES, CREATININE, OR CREATINE REPLACE HISTIDINE IN THE DIET FOR PURPOSES OF GROWTH?

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Modern nutritional investigations have shown that certain amino acids are necessary components of the diet. In the absence of these essential factors animals receiving rations adequate in other respects fail to grow. The recognition of this fact suggests the desirability of determining whether the indispensable amino acids may be replaced in the diet by other substances more or less closely related in chemical structure. Such studies would appear to offer a valuable means of discovering what types of chemical changes, particularly of the synthetic sort, may be accomplished by the living organism. It seems not improbable that compounds closely related in chemical make-up to the required amino acids might be transformed into the latter and thereby serve as growth promoters in a fashion analogous to the amino acids themselves. Moreover, inasmuch as the amino acids serve as the "Bausteine" not only in the anabolism of tissue proteins, but also in the production of many non-protein nitrogenous components of the cells such as the purines, pyrimidines, creatine, carnosine, etc., it is possible that the latter compounds might be capable, at least in part, of meeting the dietary need of the amino acids from which they come.

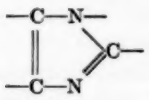
Biochemical literature affords very little information concern-

* The experimental data in this paper are taken from a thesis submitted by Gerald J. Cox in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Physiological Chemistry in the Graduate School of the University of Illinois.

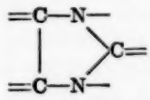
ing these problems. Perhaps the most important contribution along this line is the investigation of McGinty, Lewis, and Marvel (1924–25), in which several caproic acid derivatives were added to a diet deficient in lysine. These authors report that α-hydroxy-, ε-hydroxy-, ε-amino-, and α-hydroxy-ε-amino caproic acids are entirely unable to replace lysine in the ration of the growing animal. In an earlier paper Lewis and Root (1920) had shown that nor-leucine is incapable of replacing lysine. It appears, therefore, that the substances in question, despite their close chemical similarity to lysine, cannot be transformed into the latter by the organism.

M. L. Mitchell (1924) has published the results of feeding experiments in which taurine was added to a diet low in cystine. The author found that upon such a dietary régime young mice grow at a practically normal rate, and states that, "The evidence obtained appears to show that taurine can, in fact, take the place of a considerable proportion of the cystine in the diet." He adds, "this fact does not constitute any evidence that taurine is actually convertible into cystine in the animal body." We shall consider Mitchell's findings in the following paper.

Our interest in the nutritive importance of histidine prompted us to determine whether the functions of this amino acid may be performed by other substances. We have shown previously (Rose and Cox, 1924) that histidine belongs to the group of indispensable amino acids. It appears to be especially well suited for replacement investigations (*a*) because its exclusion from the diet is followed by prompt and very striking changes in the growth curves of the experimental animals, and (*b*) because it is rather closely related in structural configuration to several compounds of physiological interest which normally occur as components of the cells or of the excreta. Of these the purines and creatinine are of particular interest. In a recent paper from this laboratory Rose and Cook (1925) have shown that histidine is one of the precursors of purines. Evidently, the imidazole ring in the purine structure has its origin, at least in part, in this amino acid. Creatinine is a derivative of the glycocyamidine ring which may be regarded as a reduced imidazole structure (*cf.* the accompanying formulas), and hence might be related physiologically to histidine.



Imidazole ring.



Glycocyamidine ring.

In this and the following paper we are presenting the results of growth studies in which young animals received diets deficient in histidine but supplemented with compounds of more or less close similarity to the amino acid in chemical structure. In the present communication are presented the results of the purine, creatinine, and creatine investigations.

TABLE I.
Composition of Diets.

	Diet 7.	Diet 11.
	gm.	gm.
Casein	14.7	
"Deficient digest"		14.05
Cystine	0.3	0.30
Tyrosine		0.45
Tryptophane		0.20
Dextrin	40.0	40.00
Sucrose	15.0	15.00
Lard	19.0	19.00
Cod liver oil	5.0	5.00
Salt mixture	4.0	4.00
Agar	2.0	2.00
Total	100.0	100.00

EXPERIMENTAL.

White rats were used throughout as the experimental subjects. The composition of the diets is shown in Table I. Diet 7 is the normal ration which we have employed in many feeding experiments in the past. Diet 11 is adequate in every respect except as regards its histidine content. The so called "deficient digest" used in Diet 11 to supply the nitrogenous portion of the ration was prepared in a manner described in detail in the paper of Rose and Cox (1924). In the creatinine and creatine experiments the histidine was removed from the hydrolyzed casein by double

precipitation with silver sulfate and barium hydroxide. This procedure, as was pointed out by Rose and Cox (1924), renders the resulting material very low in histidine. Only the slightest color is obtained with the Hunter (1922) test. In the purine experiments it seemed desirable to have a ration containing somewhat

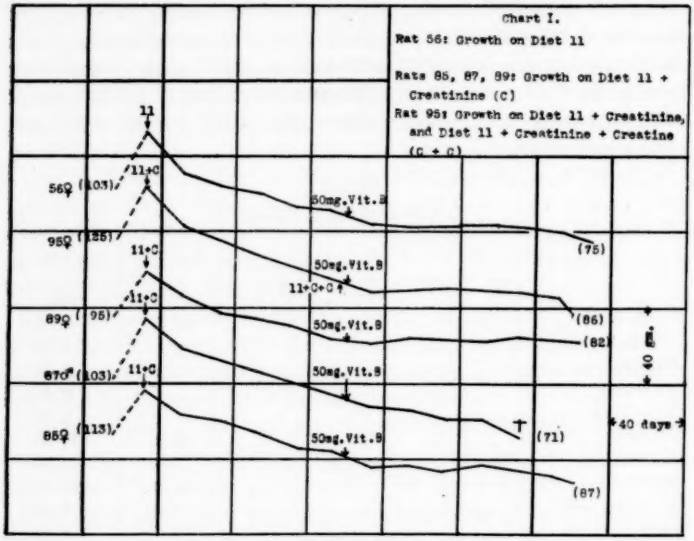


CHART I.

The broken line portions of the curves represent the growth of the rats during the preliminary periods upon a diet of whole casein (Diet 7 of Rose and Cox (1924)). The solid lines show the growth upon the histidine-deficient ration (Diet 11 of Rose and Cox (1924)), either with or without the addition of creatinine, creatine, or purines. The arrows denote the points at which the dietary changes were made. The letters indicate the supplementing agent employed in each experiment as explained in the legends on the individual charts. The numbers in parentheses signify the initial and final weights of the rats.

larger traces of histidine than are present after double precipitation. Our reason for this procedure is based upon the idea that since histidine is normally transformed in part into purines, the inclusion of the latter in the diet might spare and make available for growth the traces of histidine which otherwise might be used

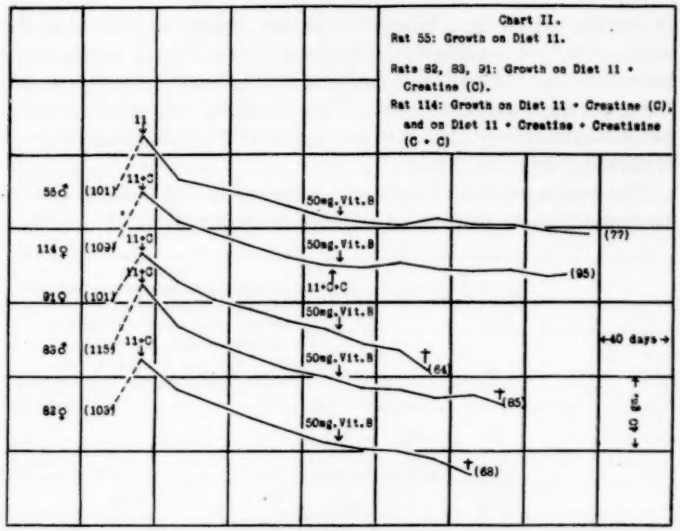


CHART II.

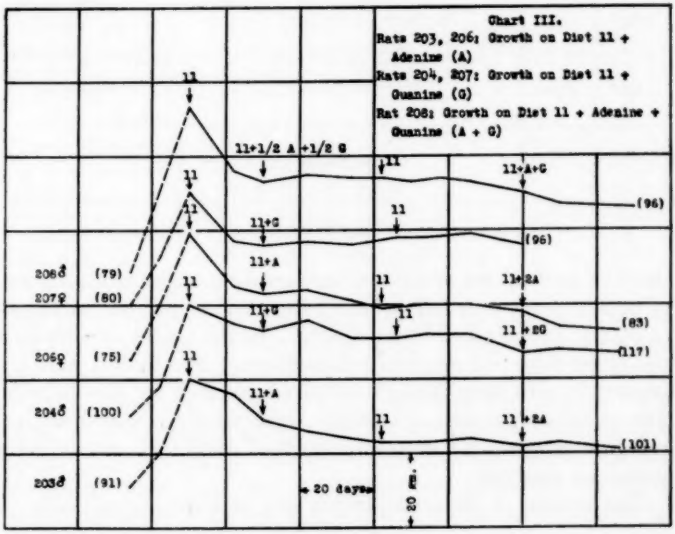


CHART III.

in purine synthesis. Therefore, in the purine experiments the deficient digest consisted of a mixture of two doubly precipitated preparations of hydrolyzed casein and two preparations which had been precipitated but once. The resulting material provided better maintenance than was possible when the histidine had been removed more completely.

The water-soluble vitamin was supplied in the form of tablets containing yeast extract mixed with dextrin and water. During

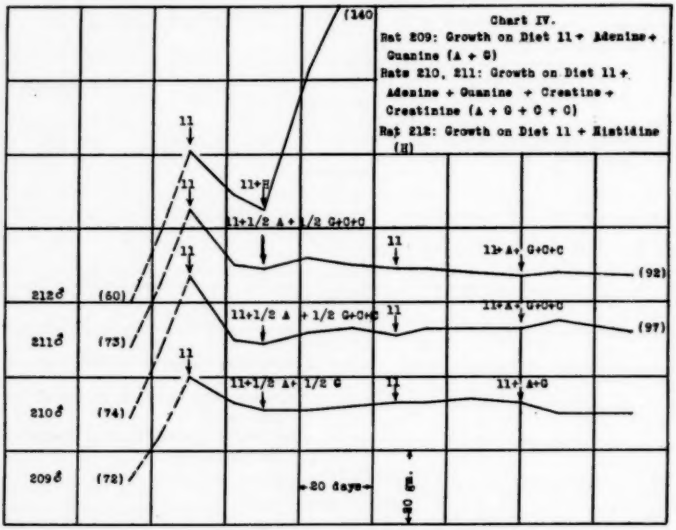


Chart IV.

the first half of the creatinine and creatine experiments 25 mg. of yeast extract were fed daily. Later this amount was increased to 50 mg. as indicated in the charts. In the purine experiments the larger dose was employed throughout. The tablets were fed separately and were always devoured greedily by the animals. The adenine, guanine, creatinine, and creatine were commercial products which had been repurified until they gave correct values on analysis.

The results of the experiments are presented graphically in

Charts I to IV. Tables II to VI inclusive show the average daily food consumption and the average daily change in body weight of each animal. The broken line portions of the curves represent the growth of the animals upon Diet 7; the unbroken lines show the growth upon Diet 11 either with or without the addition of the compounds being tested. Charts I and II indicate the effects of supplementing a histidine-deficient diet with creatinine, creatine, or both. The supplementing agents were added directly to

TABLE II.

Food Consumption and Body Weight Changes.

Days.	Diet.	Average daily change in body weight.	Average daily food consumption.	Days.	Diet.	Average daily change in body weight.	Average daily food consumption.
Rat 56 ♀.				Rat 82 ♀.			
		gm.	*gm.*			*gm.*	*gm.*
1–14	7	+2.00	8.3	1–16	7	+1.63	8.5
14–74	11	−0.50	6.2	16–72	11 + 0.85 per cent creatine.	−0.55	5.6
74–114		−0.25	4.2	72–118		−0.30	4.4
114–174		−0.13	4.4	118–154		−0.11	4.5
174–253		−0.10	4.6	154–202		−0.25	4.8
Rat 83 ♂.				Rat 85 ♀.			
1–16	7	+2.19	9.4	1–16	7	+1.38	8.4
16–72	11 + 0.85 per cent creatine.	−0.68	4.8	16–72	11 + 0.65 per cent creatinine.	−0.36	5.4
72–118		−0.30	3.6	72–118		−0.28	3.7
118–154		−0.14	3.8	118–178		−0.13	4.6
154–210		−0.14	4.3	178–246		−0.10	5.4

Diet 11. The amounts employed were 0.65 gm. of creatinine or 0.85 gm. of creatine per 100 gm. of food. Each of these quantities is equivalent to the sum of the histidine and arginine present in 100 gm. of Diet 7. When both creatinine and creatine were added, as in the experiments upon Rat 95 (Chart I) and Rat 114 (Chart II), 0.275 gm. of creatinine and 0.49 gm. of creatine per 100 gm. of food were used. Of these quantities the former is equivalent to the histidine, and the latter to the arginine present

in Diet 7. For purposes of comparison the upper curve in each of the two charts represents the growth of a rat upon exactly the same diet without the addition of creatinine or creatine.[1] It is evident that in no case was growth improved by the addition to the ration of creatinine, creatine, or both of these physiological products.

TABLE III.

Food Consumption and Body Weight Changes.

Days.	Diet.	Average daily change in body weight.	Average daily food consumption.	Days.	Diet.	Average daily change in body weight.	Average daily food consumption
Rat 87 ♂.				Rat 89 ♀.			
		gm.	gm.			gm.	gm.
1–16	7	+2.00	7.8	1–16	7	+1.50	8.3
16–72	11 + 0.65 per cent creatinine.	−0.48	5.2	16–72	11 + 0.65 per cent creatinine.	−0.43	4.8
72–118		−0.28	3.5	72–118		−0.26	3.8
118–178		−0.22	4.3	118–178		−0 02	4.4
178–218		−0.28	5.4	178–249		0	5.1
Rat 91 ♀.				Rat 95 ♀.			
1–16	7	+1.56	8.2	1–16	7	+1.75	9.1
16–72	11 + 0.85 per cent creatine.	−0.52	4.8	16–72	11 + 0.65 per cent creatinine.	−0.59	5.7
72–112		−0.28	4.2	72–118		−0.35	4.4
112–170		−0.38	4.1	118–178	11 + 0.49 per cent creatine + 0.275 per cent creatinine.	−0.07	5.2
				178–246		−0.21	5.7

The experiments with the purines were conducted in a slightly different manner. Following the normal period upon Diet 7

[1] The growth curve of one of these animals (Rat 55, Chart II) is reproduced from a paper of Rose and Cox (1926) to which the reader is referred for the average daily change in body weight and the average daily food consumption of the animal.

the rats were changed to Diet 11. This always resulted in a rather rapid loss in weight. But since the deficient digest used in the purine experiments contained, as explained above, somewhat

TABLE IV.

Food Consumption and Body Weight Changes.

Days.	Diet.	Average daily change in body weight.	Average daily food consumption.	Days.	Diet.	Average daily change in body weight.	Average daily food consumption.
Rat 114 ♀.				Rat 203 ♂.			
		gm.	*gm.*			*gm.*	*gm.*
1–16	7	+1.94	9.2	1–16	7	+1.81	7.3
16–72	11 + 0.85 per cent creatine.	−0.50	5.5	16–36	11	−0.55	4.7
72–118		−0.26	3.7	36–68	11 + 0.485 per cent adenine sulfate.	−0.19	5.2
118–178		−0.03	4.8				
178–247		−0.04	5.9	68–106	11	−0.03	4.9
				106–132	11 + 0.97 per cent adenine sulfate.	−0.04	5.2
Rat 204 ♂.				Rat 206 ♀.			
1–16	7	+1.87	7.4	1–16	7	+2.12	7.6
16–36	11	−0.35	4.4	16–36	11	−0.80	4.6
36–72	11 + 0.49 per cent guanine hydrochloride.	−0.06	4.3	36–68	11 + 0.485 per cent adenine sulfate.	−0.13	3.8
72–106	11	−0.12	4.3	68–106	11	−0.03	3.7
106–132	11 + 0.98 per cent guanine hydrochloride.	0	4.8	106–132	11 + 0.97 per cent adenine sulfate.	−0.19	4.4

larger amounts of histidine than were employed in the creatinine and creatine experiments, the animals lost weight much less rapidly after the first 20 days, and usually were in a state of approximate maintenance. When this condition had been attained

TABLE V.

Food Consumption and Body Weight Changes.

Days.	Diet.	Average daily change in body weight.	Average daily food consumption.
	Rat 207 ♀.		
		gm.	*gm.*
1–16	7	+1.88	7.6
16–36	11	−0.70	3.3
36–72	11 + 0.49 per cent guanine hydrochloride.	+0.06	3.9
72–106	11	−0.06	4.2
	Rat 209 ♂.		
1–16	7	+1.75	7.1
16–36	11	−0.45	3.4
36–72	11 + 0.24 per cent adenine sulfate + 0.245 per cent guanine hydrochloride.	+0.06	4.1
72–106	11	0	4.4
106–136	11 + 0.485 per cent adenine sulfate + 0.49 per cent guanine hydrochloride.	−0.10	3.8

Days.	Diet.	Average daily change in body weight.	Average daily food consumption.
	Rat 208 ♂.		
		gm.	*gm.*
1–16	7	+2.75	8.9
16–36	11	−1.00	4.0
36–68	11 + 0.24 per cent adenine sulfate + 0.245 per cent guanine hydrochloride.	0	4.1
68–106	11	−0.08	4.1
106–136	11 + 0.485 per cent adenine sulfate + 0.49 per cent guanine hydrochloride.	−0.13	4.0
	Rat 210 ♂.		
1–16	7	+2.38	7.4
16–36	11	−0.90	2.9
36–72	11 + 0.24 per cent adenine sulfate + 0.245 per cent guanine hydrochloride + 0.65 per cent creatinine + 0.85 per cent creatine.	+0.06	3.7
72–106	11	+0.06	3.5
106–136	11 + 0.485 per cent adenine sulfate + 0.49 per cent guanine hydrochloride + 0.65 per cent creatinine + 0.85 per cent creatine.	−0.03	3.5

the purines were added to the food. Guanine was used as the sulfate and adenine as the hydrochloride. The amount of each was calculated on the basis of the histidine present in Diet 7.

TABLE VI.

Food Consumption and Body Weight Changes.

Days.	Diet.	Average daily change in body weight.	Average daily food consumption.	Days.	Diet.	Average daily change in body weight.	Average daily food consumption.
Rat 211 ♂.				Rat 212 ♂.			
		gm.	gm.			gm.	gm.
1–16	7	+2.31	6.8	1–16	7	+2.56	7.1
16–36	11	−0.80	2.7	16–36	11	−0.80	3.3
36–72	11 + 0.24 per cent adenine sulfate + 0.245 per cent guanine hydrochloride + 0.65 per cent creatinine + 0.85 per cent creatine.	0	4.3	36–56	11 + 0.5 per cent histidine monochloride.	+2.75	6.6
72–106	11	−0.06	3.8				
106–136	11 + 0.485 per cent adenine sulfate + 0.49 per cent guanine hydrochloride + 0.65 per cent creatinine + 0.85 per cent creatine.	0	3.6				

Either one-half, one, or two equivalents were employed as denoted in the charts. In two experiments (Rats 73 and 74, Chart IV) 0.65 gm. of creatinine and 0.85 gm. of creatine per 100 gm. of food

were incorporated in the ration in addition to half an equivalent of each purine. The curves show that absolutely no improvement in growth resulted in any of the experiments from the additions in question. In striking contrast to the behavior of the rats receiving the purines is the growth curve of Rat 212 (Chart IV), to the diet of which was added 0.5 gm. of histidine monochloride per 100 gm. of food. This resulted immediately in a very rapid increase in body weight. It is evident that the purines, despite the fact that they contain the imidazole grouping, are quite unable to replace histidine in nutrition. This fact would appear to indicate that the formation of purines from histidine is an irreversible reaction in the animal body.

SUMMARY.

The results of experiments upon growing animals receiving diets deficient in histidine but adequate in every other respect to meet the nutritive requirements of the organism indicate that neither adenine, guanine, creatinine, creatine, nor a combination of these compounds is capable of functioning in place of the missing amino acid. These results are interpreted as indicating that the reaction of purine synthesis from histidine is an irreversible one in the animal organism.

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THE AVAILABILITY OF SYNTHETIC IMIDAZOLES IN SUPPLEMENTING DIETS DEFICIENT IN HISTIDINE.*

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In continuation of the investigations outlined in the preceding paper, we have studied also the possibility of replacing histidine by substances more closely related to it in chemical structure than are creatinine and the purines. For this purpose we have employed imidazole, 4-methylimidazole, 4-hydroxymethylimidazole, 4-imidazole formaldehyde, 4-imidazole carboxylic acid, 4-imidazole acetic acid, β-4-imidazole propionic acid, β-4-imidazole acrylic acid, and *dl*-β-4-imidazole lactic acid (*dl*-β-4-imidazole-α-hydroxy propionic acid). It is evident that this series of compounds includes substances both with and without side chains attached to the imidazole nucleus, and substances in which the complexity of the side chain varies from one to three carbons.

Several of the above compounds probably occur in physiological materials, or may arise under the influence of living cells. Koessler and Hanke (1924) have developed a quantitative method for the estimation of urinary imidazoles, which when applied to normal and pathological urines, indicates the excretion of 120 to 220 mg. or more daily. The nature of these compounds is, however, unknown. Theoretically, 4-imidazole acetic acid might arise as a product of histidine catabolism through oxidation of the

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† The experimental data in this paper are taken from a thesis submitted by Gerald J. Cox in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Physiological Chemistry in the Graduate School of the University of Illinois.

corresponding ketonic acid, provided the latter is produced in histidine deamination. Ackermann (1910) isolated what he believed to be β-4-imidazole propionic acid as a result of long continued action of bacteria on histidine. According to Hunter (1912) β-4-imidazole acrylic acid is identical with urocanic acid, isolated first by Jaffe (1874, 1875) from the urine of the dog, and later by Swain (1905) from the urine of the coyote. Raistrick (1917) separated urocanic acid from putrefaction mixtures containing histidine, while Kotake and Konishi (1922) report the isolation of the same substance from the urines of a dog after feeding or injecting large amounts of histidine. On the basis of perfusion experiments Konishi (1922) reached the conclusion that urocanic acid is a normal intermediate in histidine catabolism.

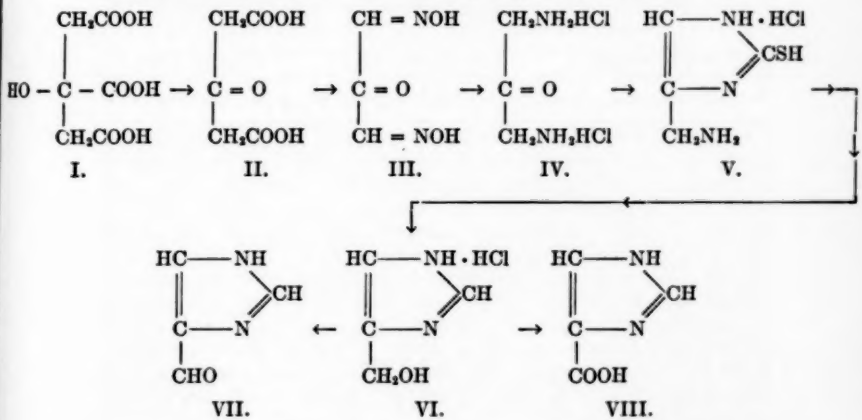
The above incomplete review of the literature will suffice to indicate that any or all of the imidazoles employed by us may bear a more or less close relationship to histidine metabolism. On the contrary, the recognition of this fact does not imply that the synthesis of histidine could be accomplished from any of the compounds in question. If the imidazole nucleus were the only stumbling block in the path of histidine synthesis in the organism, then the inclusion in the ration of imidazole itself, or any of its simpler derivatives, might serve to render a histidine-deficient diet adequate for purposes of growth. On the other hand, the organism may be much more exacting in its dietary demands, and require the presence not only of the imidazole ring, but also of a side chain of definite structure attached to the nucleus in the proper position. Again, the synthesis of histidine in the body might be impossib'e of attainment no matter what substances were furnished in the diet, or how closely they were related to the amino acid in chemical structure. Under such circumstances histidine would be indispensable even when the imidazoles in question were supplied. The following experiments were undertaken for the purpose of obtaining additional information concerning these points.

EXPERIMENTAL.

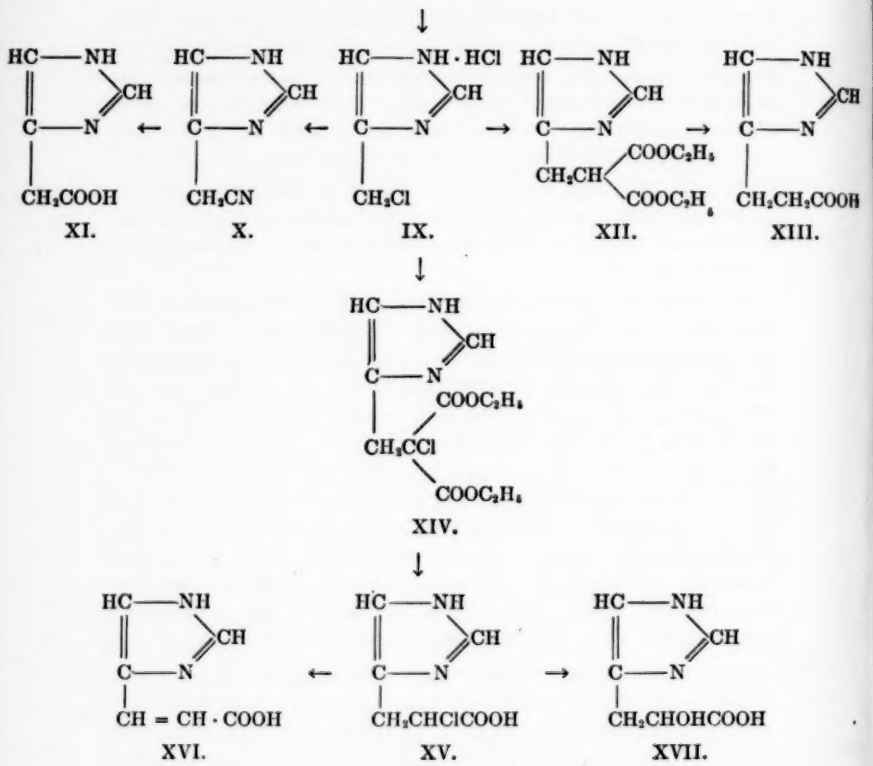
The methods of procedure were identical with those employed in the preceding paper, and need not be repeated here. The "deficient digest" used in Diet 11 was uniform for all of the experiments herein reported, and consisted of two doubly precipitated preparations of hydrolyzed casein mixed thoroughly with two preparations which had been precipitated but once. The resulting material, when supplemented with the other necessary ingredi-

ents of a normal diet, failed to permit growth, but provided better mainten nce than did diets in which the histidine had been reduced to a lower level. This procedure appeared to offer a delicate means of detecting replacement of histidine by other imidazoles, since the inclusion in the diet of a compound capable of substituting for histidine would probably manifest effects on the growth rates more promptly than if all of the amino acid had been removed. Numerous control experiments showed that the addition of histidine sufficed to render the ration capable of supporting rapid and sustained growth.

The greatest difficulty encountered in the investigation was in connection with the preparation of the imidazoles. These were all synthesized in this laboratory by methods which, with slight modifications, are described in the literature.[1] Imidazole itself was prepared essentially according to the directions of Fargher and Pyman (1919); 4-methylimidazole by the method of Windaus and Knoop (1905) as modified by Koessler and Hanke (1919). The imidazole and 4-methylimidazole so obtained melted at 88° and 55°C., respectively. These melting points are quite close to those recorded in the literature for these substances. The other imidazoles were prepared from 4-hydroxymethylimidazole. The reactions involved in its synthesis, and in the derivation of the other compounds from it, are schematically represented below.



[1] The authors are indebted to Prof. C. S. Marvel of the Organic Division for many valuable suggestions in connection with the synthetic procedures.



Starting with citric acid (I), acetone dicarboxylic acid (II) was prepared according to the directions of Ingold and Nickolls (1922). After filtering off this compound on filtros plates, and sucking as dry as possible, it was used directly in the preparation of diaminoacetone dihydrochloride (IV) by treating with sodium nitrite and reducing the resulting diisonitroso acetone (III) with stannous chloride and hydrochloric acid. From the diaminoacetone dihydrochloride, 4-hydroxymethylimidazole (VI) was prepared through the intermediate 2-thiol-4-aminomethylimidazole hydrochloride (V) according to the procedure of Pyman (1911, *a*, *b*), as modified in certain particulars by Koessler and Hanke (1918). The resulting product melted at 107–108°C. (uncorrected). Koessler and Hanke (1918) report a melting point of 107–109°C. The yields in the several steps varied from 23 to 89 per cent of the theoretical.

4-Imidazole formaldehyde (VII) and 4-imidazole carboxylic acid (VIII)

were prepared simultaneously by the oxidation of 4-hydroxymethylimidazole with concentrated nitric acid (Pyman, 1916). The two derivatives were separated readily by rendering their concentrated solution alkaline with sodium carbonate, under which condition the aldehyde crystallized. This product melted at 170°C. On faintly acidifying the filtrate with hydrochloric acid, 4-imidazole carboxylic acid (melting at 283°C. on the Maquenne block) crystallized.

4-Imidazole acetic acid (XI) was obtained in an attempt to prepare β-4-imidazole pyruvic acid by condensation of 4-cyanomethylimidazole (X) with diethyl oxalate, and subsequent hydrolysis of the resulting ester. 4-Chloromethylimidazole hydrochloride and 4-cyanomethylimidazole were prepared according to the methods of Pyman (1911, *a*). The subsequent steps in the process were carried out as follows: 2.0 gm. of sodium were added to 30 cc. of absolute alcohol in a 200 cc. round bottomed flask, and heated under a reflux condenser until all had dissolved. 13.5 gm. of diethyl oxalate and 9.2 gm. of solid 4-cyanomethylimidazole were added. The flask was stoppered and left overnight. Then 12 cc. of water were added, and 8.6 gm. of concentrated hydrochloric acid (sp. gr. 1.19). On cooling, long needles separated which were filtered off and washed with cold water. The substance was found to be fairly soluble in hot water or alcohol, but insoluble in ether, petroleum ether, chloroform, or acetone.

To recrystallize, all of the material was warmed on a steam cone with 300 cc. of water. Only a part of it dissolved. After filtering off the insoluble material, the apparently saturated filtrate yielded fine brown needles on cooling. These crystals, after drying, darkened at 215° and melted at 253°C. (uncorrected). Analysis of the material by the Kjeldahl method showed 19.60 per cent nitrogen as compared with a calculated 20.29 per cent for ethyl-β-cyano-β-4-imidazole pyruvate. Inasmuch as this material did not yield the expected imidazole pyruvic acid in the subsequent hydrolysis with acid, but imidazole acetic acid instead, we cannot be certain that ethyl-β-cyano-β-4-imidazole pyruvate was obtained. In an attempt to recrystallize the main bulk of the substance, nearly 2000 cc. of water at 80°C. were necessary to effect solution. On cooling no crystals separated. On concentrating to about 200 cc. no crystallization occurred, even after cooling and seeding.

After this failure to obtain crystals as in the preliminary test, it was decided to proceed with the original plan of hydrolysis of the cyano ester in the hope that β-4-imidazole pyruvic acid might be obtained. Accordingly, 50 cc. of concentrated sulfuric acid were added to 200 cc. of the solution, and the resulting clear yellow liquid was boiled for 3 hours. It was then cooled and exactly neutralized with barium hydroxide. The barium sulfate was filtered off, and the solution concentrated until crystallization started. Fine, glistening, white needles formed. After a single recrystallization from water the substance melted at 223°C. (uncorrected). Two determinations of nitrogen by the Kjeldahl method gave 19.22 and 19.27 per cent respectively. The theory for β-4-imidazole pyruvic acid is 18.18

per cent and for 4-imidazole acetic acid is 19.44 per cent nitrogen. The melting points recorded in the literature for 4-imidazole acetic acid are 220°C. (Knoop, 1907), 222°C. (corrected) (Pyman, 1911, *a*), and 226–228°C. (corrected) (Koessler and Hanke, 1918). It is evident, therefore, that 4-imidazole acetic acid, instead of β-4-imidazole pyruvic acid, was secured in the above series of reactions.

β-4-Imidazole propionic acid (XIII) was made from 4-chloromethyl-imidazole by condensation with diethyl malonate and subsequent saponification as described by Pyman (1911, *b*). The resulting product melted at 208–209°C. (uncorrected).

β-4-Imidazole acrylic (urocanic) acid (XVI) and *dl*-β-4-imidazole lactic acid (XVII) were prepared through the intermediate *dl*-α-chloro-β-4-imidazole propionic acid (XV). The latter was prepared according to the directions of Pyman (1911, *b*). For the transformation of *dl*-α-chloro-β-4-imidazole propionic acid into the acrylic acid derivative two slightly different procedures were followed. In a paper dealing with the structure of ergothioneine, Barger and Ewins (1911) state that a synthetic specimen of β-4-imidazole acrylic acid was obtained in small quantity by the action of trimethylamine on α-chloro-β-4-imidazole propionic acid. On the basis of this statement we proceeded as follows: 12 gm. of α-chloro-β-4-imidazole propionic acid were dissolved in 83 cc. of 18 per cent aqueous trimethylamine. After standing overnight at 37°C. the solution was concentrated to 40 cc., and 40 gm. of potassium hydroxide were added. The solution was boiled under a reflux condenser for 30 minutes. It was then diluted with 100 cc. of water, and treated with sufficient concentrated nitric acid to provide one-third of a volume excess. On cooling, 0.82 gm. of a crystalline nitrate (melting point 213–215°C.) was obtained. By adding one equivalent of dry sodium carbonate this was converted into 0.57 gm. of β-4-imidazole acrylic acid, which melted at 225–227°C. (uncorrected).

In a second attempt to prepare urocanic acid, 35 gm. of a syrup of *dl*-α-chloro-β-4-imidazole propionic acid hydrochloride were treated with 230 cc. of an 18 per cent aqueous trimethylamine solution and 66 gm. (one equivalent) of 10 per cent sodium hydroxide solution. After standing 2 days at 37°C., the solution was boiled for 30 minutes under a reflux condenser. It was then evaporated *in vacuo* to about 100 cc. Fine needles crystallized out, and after recrystallizing from water, 3.2 gm. of a product melting at 228–231°C. were obtained. From the combined mother liquors 3.98 gm. of the nitrate were secured by adding one-half volume of concentrated nitric acid. The yield was 26 per cent of the theoretical.

A mixture of the preparations obtained by the two methods melted at the same point (228°C. uncorrected) as separate samples in the same bath. On the Maquenne block, a melting point of 234–235°C. was obtained. The previously observed melting points of urocanic acid vary widely as shown by the following data gathered from the literature:

	°C.
Jaffe (1874)	212–213
Siegfried (1897-98)	229

Swain (1905)	208, decomposed at 220.
Barger and Ewins (1911)	235–236 (corrected).
Hunter (1912)	231–232 (")
Raistrick (1917)	226–230
Kotake and Konishi (1922)	218–220

A determination of total nitrogen by the Kjeldahl method gave 19.60 per cent as compared with the theoretical for $C_6H_8N_2O_3$ of 20.29 per cent. Some of the substance mixed with β-4-imidazole lactic acid gave an indefinite melting point at about 195°C., indicating that a simple replacement of the chlorine of the α-chloro acid by hydroxyl had not taken place.

Pyman (1911, *b*) does not give specific directions for the preparation of *dl*-β-4-imidazole lactic acid, stating only that it was formed by the action of silver hydroxide on a hot aqueous solution of α-chloro-β-4-imidazole

TABLE I.

Imidazoles Employed per 100 Gm. of Food.

	Equivalent to 0.5 gm. of histidine monochloride + H_2O.	Equivalent to 1.0 gm. of histidine monochloride + H_2O.
	gm.	*gm.*
Imidazole	0.163	0.326
4-Methylimidazole	0.196	0.392
4-Hydroxymethylimidazole hydrochloride	0.320	0.640
4-Imidazole formaldehyde	0.229	0.458
4- " carboxylic acid	0.267	0.534
4- " acetic acid + H_2O	0.345	0.690
β-4-Imidazole propionic acid	0.335	0.670
β-4- " acrylic acid	0.330	0.660
dl-β-4-Imidazole lactic acid + H_2O	0.415	0.830

propionic acid. For our purpose the following procedure was employed: 7.5 gm. of α-chloro-β-4-imidazole propionic acid were dissolved in 150 cc. of water and boiled 1 hour with the silver carbonate obtained from 9 gm. of silver nitrate. The precipitated silver salts were filtered off, and the dissolved silver removed from the filtrate by passing in hydrogen sulfide. On evaporating the filtrate from the precipitated silver sulfide, 1.7 gm. of *dl*-β-4-imidazole lactic acid crystallized. An additional 0.6 gm. was obtained from the mother liquor. The total, 2.3 gm., represents a yield of 31 per cent. It melted at 221°C. (uncorrected). Pyman (1911, *b*) reports a melting point of 222°C. (corrected).

The rats used in the experiments were allowed to attain weights of about 80 to 85 gm. each before being restricted to the synthetic

rations. They were then placed upon Diet 7 for preliminary periods, and when normal growth curves had been secured, were changed to Diet 11. This change invariably resulted in a rapid decline in weight accompanied by a marked reduction in food

TABLE II.

Food Consumption and Body Weight Changes.

Days.	Diet.	Average daily change in body weight.	Average daily food consumption.	Days.	Diet.	Average daily change in body weight.	Average daily food consumption.
	Rat 213 ♂.				Rat 214 ♂.		
		gm.	*gm.*			*gm.*	*gm.*
1–16	7	+2.56	7.1	1–16	7	+2.12	7.1
16–36	11	−0.70	3.1	16–36	11	−0.70	3.3
36–72	11 + 0.163 per cent imidazole.	−0.06	3.5	36–64	11 + 0.267 per cent imidazole carboxylic acid.	+0.07	3.5
72–92	11 + 0.326 per cent imidazole.	−0.05	3.3	64–84	11 + 0.534 per cent imidazole carboxylic acid.	+0.10	3.9
	Rat 215 ♂.				Rat 216 ♀.		
1–16	7	+2.62	8.1	1–16	7	+1.56	6.4
16–36	11	−0.80	3.6	16–36	11	−0.60	3.4
36–60	11 + 0.196 per cent methylimidazole.	−0.21	4.0	36–60	11 + 0.196 per cent methylimidazole.	−0.04	3.4
60–84	11 + 0.392 per cent methylimidazole.	−0.13	4.0	60–84	11 + 0.392 per cent methylimidazole.	−0.08	3.4

consumption as a consequence of the histidine deficiency. After the first 20 days, the losses in weight were always much less marked, and a condition approximating maintenance occurred. Then the imidazoles under investigation were added to Diet 11.

and the effects upon body weight noted. In a previous paper Rose and Cox (1924) have shown that the addition of 0.5 gm. of histidine monochloride to 100 gm. of Diet 11 renders the food adequate to support normal growth. This quantity has been used,

TABLE III.

Food Consumption and Body Weight Changes.

Days.	Diet.	Average daily change in body weight.	Average daily food consumption.	Days.	Diet.	Average daily change in body weight.	Average daily food consumption.
Rat 219 ♀.				Rat 221 ♂.			
		gm.	*gm.*			*gm.*	*gm.*
1–16	7	+1.63	6.1	1–16	7	+3.00	8.5
16–36	11	−0.45	4.5	16–36	11	−1.15	2.9
36–60	11 + 0.229 per cent imidazole formaldehyde.	0	3.8	36–60	11 + 0.32 per cent hydroxymethylimidazole.	−0.13	3.5
60–84	11 + 0.458 per cent imidazole formaldehyde.	−0.04	3.7	60–84	11 + 0.64 per cent hydroxymethylimidazole.	0	3.7
Rat 222 ♂.				Rat 231 ♂.			
1–16	7	+2.06	7.6	1–12	7	+3.50	8.4
16–36	11	−0.65	3.6	12–36	11	−0.71	3.3
36–60	11 + 0.229 per cent imidazole formaldehyde.	−0.04	3.9	36–64	11 + 0.33 per cent urocanic acid.	−0.04	3.7
60–84	11 + 0.458 per cent imidazole formaldehyde.	0	4.1	64–88		−0.04	3.6

therefore, as a basis in calculating the amount of each imidazole to be employed. The actual quantities equivalent to 0.5 gm. and 1.0 gm., respectively, of histidine monochloride are shown in Table I.

The results of the experiments are presented in Charts I to V. Tables II to VI inclusive show the average daily change in body weight and the average daily food consumption of each rat.

TABLE IV.

Food Consumption and Body Weight Changes.

Days.	Diet.	Average daily change in body weight.	Average daily food consumption.	Days.	Diet.	Average daily change in body weight.	Average daily food consumption.
Rat 232 ♂.				Rat 235 ♀.			
		gm.	gm.			gm.	gm.
1–12	7	+3.92	8.3	1–12	7	+2.84	8.3
12–36	11	−1.00	3.2	12–36	11	−0.63	3.5
36–64	11 + 0.33 per cent urocanic acid.	+0.04	3.7	36–64	11 + 0.335 per cent imidazole propionic acid.	0	3.5
64–88		−0.08	3.6	64–80		0	3.6
Rat 233 ♂.				Rat 234 ♀.			
1–12	7	+3.17	8.4	1–12	7	+3.50	8.0
12–36	11	−0.71	3.1	12–36	11	−1.21	2.6
36–44	11 + 0.415 per cent *dl*-imidazole lactic acid.	+0.50	3.9	36–44	11 + 0.415 per cent *dl*-imidazole lactic acid.	+0.75	4.0
44–76	11 + 0.83 per cent *dl*-imidazole lactic acid.	+1.13	5.1	44–76	11 + 0.83 per cent *dl*-imidazole lactic acid.	+0.69	4.2
76–112		+0.50	4.8	76–112		+0.44	4.3
112–128	11 + 0.415 per cent *l*-imidazole lactic acid.	+0.38	5.6	112–140	11 + 0.5 per cent histidine monochloride.	+0.50	6.1
128–156		−0.64	3.6	140–156		−0.06	3.7

The effects of additions of imidazole, or its single carbon derivatives, are presented in Charts I and II. In most cases a single equivalent of the imidazole calculated, as stated above, on the

basis of 0.5 gm. of histidine monochloride, was added initially. This amount was later doubled. In some of the experiments, as indicated in the charts, the larger quantities were used throughout. Neither imidazole, 4-methylimidazole, 4-hydroxymethylimidazole, 4-imidazole formaldehyde, nor 4-imidazole carboxylic acid was able to replace histidine to the slightest degree. In each case the growth curve shows either a continued maintenance or a slight tendency for the rat to decrease in body weight.

TABLE V.

Food Consumption and Body Weight Changes.

Days.	Diet.	Average daily change in body weight.	Average daily food consumption.	Days.	Diet.	Average daily change in body weight.	Average daily food consumption.
Rat 236 ♂.				Rat 237 ♂.			
		gm.	gm.			gm.	gm.
1–12	7	+2.25	6.9	1–12	7	+2.58	7.8
12–36	11	−0.92	3.2	12–36	11	−1.04	3.0
36–56	11 + 0.326 per cent imidazole.	−0.30	3.5	36–56	11 + 0.534 per cent imidazole carboxylic acid.	0	3.7
56–80		−0.08	4.2	56–80		−0.04	4.0
Rat 238 ♀.				Rat 239 ♂.			
1–16	7	+2.12	7.3	1–16	7	+2.25	7.6
16–36	11	−0.95	3.9	16–36	11	−0.65	3.7
36–56	11 + 0.345 per cent imidazole acetic acid.	−0.10	3.5	36–56	11 + 0.345 per cent imidazole acetic acid.	−0.10	3.6
56–80		−0.08	3.5	56–80		−0.13	3.4

Similar results were obtained in the experiments with the imidazole derivatives of acetic, propionic, and acrylic acids (*cf.* Chart III). The inclusion of a single equivalent of either of these compounds entirely failed to improve the quality of the ration for growth purposes. When one recalls the close similarity in chemical structure between histidine on the one hand, and the

imidazole derivatives of propionic and acrylic acids on the other, one is impressed with the fact that the organism is exceedingly exacting in its demands. It is evident that the removal from histidine of a molecule of ammonia, or the replacement of the amino

TABLE VI.

Food Consumption and Body Weight Changes.

Days.	Diet.	Average daily change in body weight.	Average daily food consumption.	Days.	Diet.	Average daily change in body weight.	Average daily food consumption.
Rat 241 ♀.				Rat 242 ♀.			
		gm.	*gm.*			*gm.*	*gm.*
1–24	7	+1.75	6.2	1–24	7	+1.50	5.7
24–52	11	−0.32	3.5	24–52	11	−0.29	3.8
52–72	11 + 0.83 per cent *dl*-imidazole lactic acid.	+0.61	4.8	52–72	11 + 0.83 per cent *dl*-imidazole lactic acid.	+0.90	5.4
72–104		+0.47	4.3	72–92		+0.45	5.1
104–124	11	−0.80	2.6				
Rat 243 ♀.				Rat 244 ♀.			
1–24	7	+1.63	6.6	1–28	7	+1.25	5.7
24–52	11	−0.54	3.8	28–52	11	−0.29	3.5
52–72	11 + 0.83 per cent *dl*-imidazole lactic acid.	+0.35	4.8	52–88	11 + 0.83 per cent *dl*-imidazole lactic acid.	+0.92	5.5
72–104		+0.56	4.3	88–114		+0.58	5.2
				114–124	11 + 0.415 per cent *l*-imidazole lactic acid.	+0.40	5.0
				124–148		−0.29	3.3

group with a hydrogen, is quite sufficient to transform an indispensable component of the diet into a compound which is absolutely worthless for purposes of growth.

In striking contrast to the above are the results of the experiments with *dl*-β-4-imidazole lactic acid (*cf.* Charts IV and V).

In each of the six rats which received this compound, its introduction into the ration occasioned a pronounced and immediate change in the growth curve. As shown in the charts, the animals were all losing weight or were in a condition of maintenance at the time

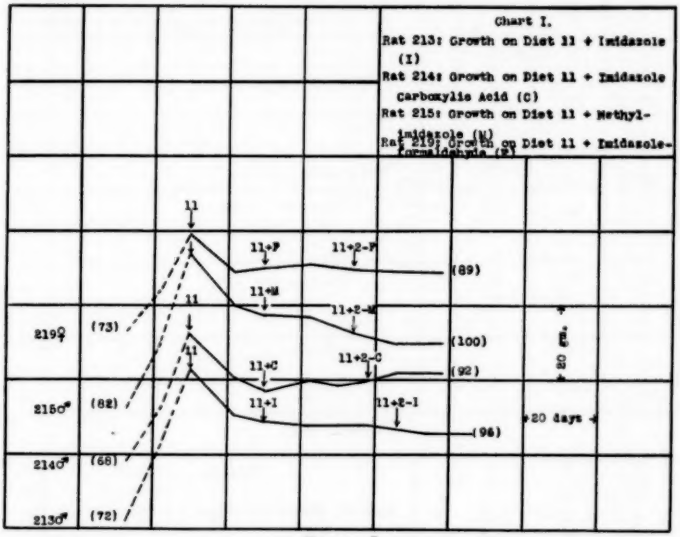


CHART I.

The broken line portions of the curves represent the growth of the animals during the preliminary periods upon a diet of whole casein (Diet 7 of Rose and Cox (1924)). The solid lines show the growth upon the histidine-deficient ration (Diet 11 of Rose and Cox (1924)), either with or without the addition of imidazoles. The arrows denote the points at which the dietary changes were made. The imidazole employed in each case is indicated by a letter as explained by the legends on the individual charts. An amount of the synthetic compound equivalent to 0.5 gm. of histidine was always used unless the figure 2 precedes the letter, in which case twice this amount was employed. The numbers in parentheses signify the initial and final weights of the rats.

dl-β-4-imidazole lactic acid was added to the food. Immediately upon receiving this substance growth began and continued to the end of the experiments, unless subsequent dietary alterations were made. Four of the six rats received two equivalents of *dl*-β-4-

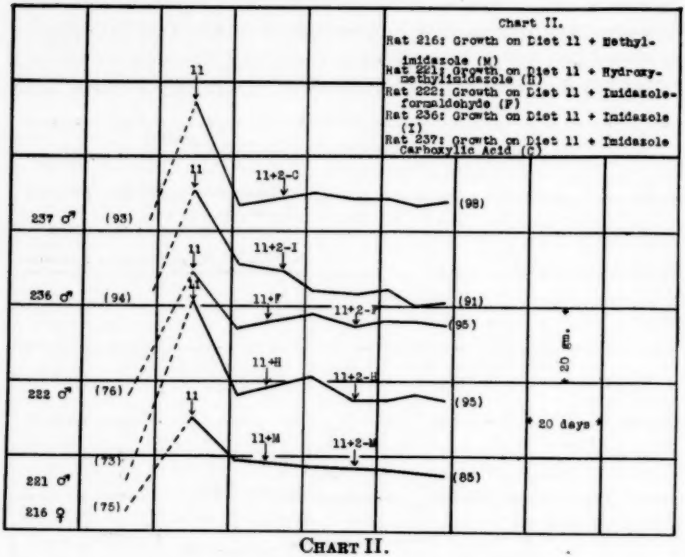


CHART II.

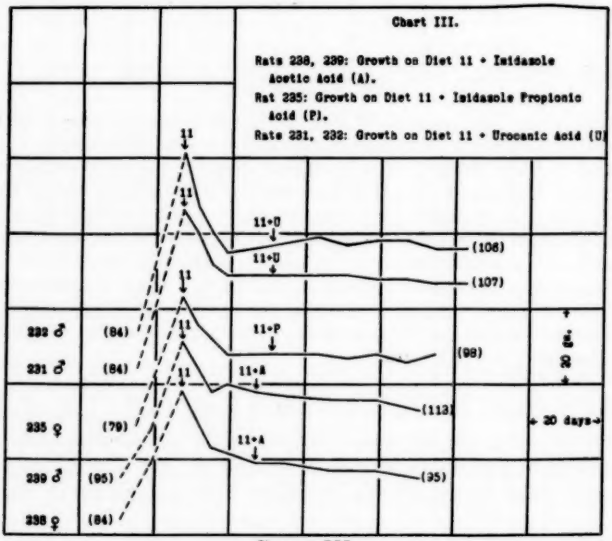


CHART III.

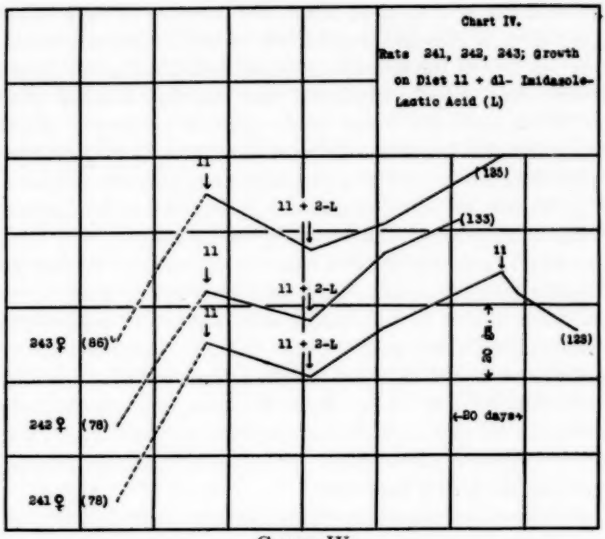


CHART IV.

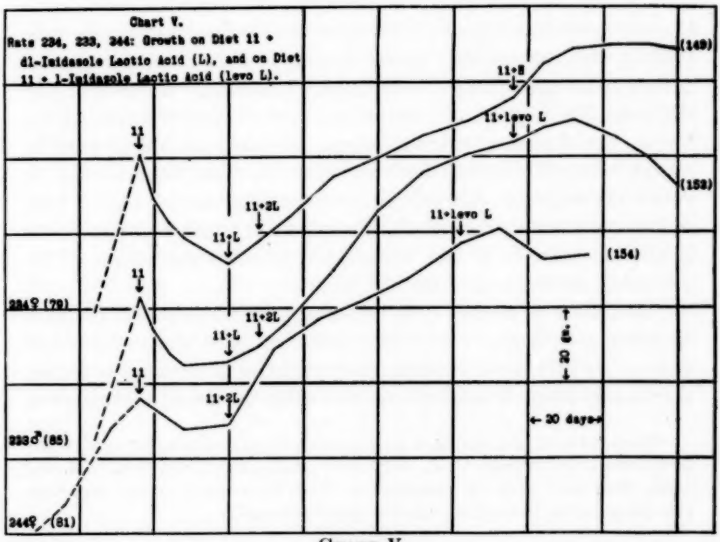


CHART V.

imidazole lactic acid so as to insure the presence of each enantiomorphic form in amount equivalent to the histidine present in casein. In two of the animals, a single equivalent of the racemic compound was initially employed, but this was doubled after 8 days. Even upon the lower intake growth occurred. Further evidence that the imidazole lactic acid was really responsible for the growth is shown by the experiment upon Rat 241 (Chart IV). During the last 20 days this animal was deprived of the lactic acid derivative and in consequence rapidly lost weight.

Two short experiments were made in an attempt to determine the active enantiomorph. Rats 233 and 244 (Chart V) were placed upon a diet to which had been added one equivalent of *l*-β-4-imidazole lactic acid. Both animals continued to grow for short periods and then lost weight rather rapidly. Obviously, the experiments were of too short duration, and the results too inconsistent to prove anything concerning the active isomer involved in growth stimulation. We expect to repeat this part of the investigation at a later date.

The above experiments constitute the first successful attempt by means of growth studies, to replace an "indispensable" amino acid by any other compound whatsoever. A single possible exception to this statement is to be found in the recent paper of Mitchell (1924), who reports that a diet low in cystine can be rendered adequate for growth by the addition of taurine. In view of the chemical dissimilarity of taurine and cystine such a substitution, if true, would be most extraordinary. We are now endeavoring to repeat Mitchell's experiments and thus far have been unable to verify his findings. Certainly, our experience shows that if taurine can replace cystine at all, the extent to which this can occur is not comparable to the remarkable substituting action of *dl*-β-4-imidazole lactic acid for histidine.[2]

The ability of animal cells to form α-amino acids from corresponding α-hydroxy or α-ketonic acids has been known for many years. In 1911, after feeding phenyl-α-ketobutyric acid to a dog, Knoop and Kertess isolated from the urine the acetyl derivative of

[2] Since writing the above a preliminary report of similar studies by Lewis and Lewis (Lewis, G. T., and Lewis, H. B., *Proc. Soc. Exp. Biol. and Med.*, 1926, xxiii, 359) has appeared, in which the authors report that they also were unable to confirm the findings of Mitchell.

d-phenyl-α-aminobutyric acid. By means of liver perfusion experiments, Embden and Schmitz (1910, 1912) showed that alanine, phenylalanine, and tyrosine can be formed from the ammonium salts of pyruvic, phenyl pyruvic, and *p*-hydroxyphenyl pyruvic acids respectively. They observed the synthesis of alanine from lactic acid also. Using a similar technique Kondo (1912) has reported the synthesis of α-aminobutyric acid and norleucine from the corresponding ketonic acids. It does not necessarily follow, however, that such a formation of an amino acid could proceed indefinitely, or at a sufficiently rapid rate to meet the growth requirements of a young animal. Indeed, attempts by Abderhalden (1915–16, 1922) to replace tyrosine, phenylalanine, and leucine of the diet by the appropriate ketonic acids completely failed. It is interesting, therefore, to note that in the long continued experiments herein reported, involving practically normal conditions of diet, the synthesis of histidine from the corresponding hydroxy acid must have occurred daily through periods of many weeks. Furthermore, our results appear to indicate that the term indispensable as applied to amino acids is a relative one, and merely involves the fact that under the usual conditions of diet the amino acids in question cannot be synthesized. Under the same dietary conditions dispensable amino acids, such as glycocoll, can be synthesized. Apparently the distinction between the two classes of amino acids is dependent solely upon the ability of the organism to accomplish their synthesis out of the materials ordinarily available.

The apparent contradiction between our results and those of McGinty, Lewis, and Marvel (1924–25) with the hydroxy derivatives corresponding to lysine, may be due to a difference in ease or speed of formation of the two amino acids in question. Possibly the synthesis of lysine from α-hydroxy-ϵ-aminocaproic acid is a slower or more difficult process for the cells than is the formation of histidine from imidazole lactic acid. The possibility of replacing other so called indispensable amino acids by synthetic compounds more or less closely related to the former in chemical structure involves problems of considerable biochemical interest. Such studies are already under way in this laboratory.

SUMMARY.

1. Studies have been made of the influence upon growth of synthetic compounds containing the imidazole ring. For this purpose the substances in question were added to a basal diet in which the nitrogen was supplied in the form of completely hydrolyzed casein from which most of the histidine had been removed by precipitation with silver sulfate and barium hydroxide. No influence upon the growth of rats was observed following the inclusion in the ration of imidazole, 4-methylimidazole, 4-hydroxymethylimidazole, 4-imidazole formaldehyde, 4-imidazole carboxylic acid, 4-imidazole acetic acid, β-4-imidazole propionic acid, or β-4-imidazole acrylic acid. One or two equivalents of either of the above substances entirely failed to inhibit the loss in weight occasioned by the deficiency of histidine.

2. On the other hand, the addition of *dl*-β-4-imidazole lactic acid to the histidine-deficient diet caused an immediate resumption of growth, at a rate slightly slower than that induced by the equivalent quantity of histidine. It is evident that under the conditions of the experiments the synthetic product in question is capable of serving in place of histidine, probably through being transformed by the cells into the amino acid.

3. The above results constitute the first successful attempt, by means of growth experiments, to replace an indispensable amino acid of the diet by a non-amino compound.

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ANIMAL CALORIMETRY.

THIRTY-SECOND PAPER.

THE PHYSIOLOGICAL BEHAVIOR OF GLUCOSANE.*

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The discovery of a carbohydrate which is capable of oxidation in the diabetic would be second only in importance to the discovery of insulin. Such a substance would necessarily be antiketogenic and not give rise to any extra urinary glucose. The compounds which might be suspected of such behavior would be the intermediary products of carbohydrate metabolism, if it be assumed that the function of insulin is to change glucose into a readily oxidizable intermediate product, which physiological process is not present in the diabetic.

As early as 1914 Grafe (1) proposed the use of caramel as affording a source of utilizable carbohydrate for the diabetic. Experiments of this investigator show that a decreased acidosis was concomitant with caramel administration while the glucosuria was not greatly increased. In some cases gastrointestinal disturbances and diarrhea resulted, especially when the caramel was given in large amounts and over long periods of time. The antiketogenic effects of caramel or of caramelized breads—prepared by heating at high temperatures—has more recently been attributed by Grafe (2) to a commercial oatmeal preparation,

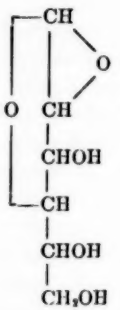
* A preliminary report of this work has been published in *Proc. Soc. Exp. Biol. and Med.*, 1926, xxiii, 431.

† Shangtung Christian University, Medical Fellow of the Rockefeller Foundation, 1925-26.

"Satrose," a product without the unpalatableness of the caramelized pastries and not causing the gastrointestinal disturbances which caramel does.

Grafe and von Schröder (3) have recently amplified their earlier observations and now attribute the favorable effects of caramel and caramelized pastries and breads to the anhydrosugar, glucosane, which is presumably present in caramel and which, according to these investigators, behaves exactly as caramel does. Experiments with glucosane have likewise been carried out by Kerb and Kerb-Etzdorf (4), who conclude that this substance is probably more readily convertible into glycogen than glucose, rather than that it is directly oxidizable. Such results are also reported by Nonnenbruch (5) and Nothmann and Kühnau (6). Kerb and Schilling (7) report that "Salabrose," a tetraglucosane preparation, is not eliminated in the urine, that it is absorbed, and is of value in the treatment of diabetes.

Glucosane, which is non-fermentable and not attacked by *Bacillus coli*, has the following formula according to Cramer and Cox (8):



It does not reduce copper solutions, but is readily changed to glucose by boiling with dilute acids (9).

The same properties as those ascribed to glucosane and caramel are claimed for other substances. Thannhauser and Jenke (10) have attributed to glucosone antiketogenic properties when given to diabetics, while in this country Rabinowitch (11) has claimed that dihydroxyacetone is utilizable in the diabetic, the latter result being vigorously denied by Campbell, Fletcher, Hepburn,

and Markowitz (12). Dakin, Benedict, and West (13) have recently shown the inability of the normal and diabetic to utilize the hexose, glutose.

In view of the wide-spread use of glucosane in the treatment of diabetes in Germany, it seemed of importance to repeat the investigations on animals made diabetic with phlorhizin so that we might determine to what extent this substance, administered orally, is absorbed and oxidized, as judged by the effect on the respiratory quotient, what effect it exerts on the glucosuria, and its fate after subcutaneous and intraperitoneal injection.

Methods.

The experiments reported here were carried out on six dogs, three of which were phlorhizinized and three of which were normal. The animals were made diabetic by the usual technique of this laboratory; namely, by the daily subcutaneous injection of 1 gm. of phlorhizin in 10 cc. of olive oil.

The glucosane used in some of the early experiments was prepared by heating glucose *in vacuo* at 160–170° for 1 to 2 hours in an oil bath according to the method of Pictet and Gasten (14). This method did not result in a quantitative conversion of glucose to glucosane, 30 to 50 per cent of free glucose usually remaining unchanged. In one case by this procedure all except 15 per cent of the glucose was changed to glucosane. The majority of the experiments were carried out with the glucosane prepared in Germany by the Chemische Werke Grenzach A. G. under the trade name of "Salabrose."

Urinary glucose was determined by the Bertrand method since the highly colored glucosane solutions could not be determined by the Benedict procedure. Total nitrogen was determined by the Kjeldahl method.

Since no procedure was available for the qualitative or quantitative determination of glucosane, it was necessary to devise one. It was found that on boiling with dilute acid quantitative conversion of this substance to glucose resulted, which after neutralization could then be determined by the usual analytical methods for glucose. Table I shows the results obtained with salabrose when a solution of this substance was boiled with water and with different concentrations of HCl.

Boiling with water for 15 or 30 minutes was without effect on the glucosane, while the maximum conversion to glucose occurred on boiling with 5 per cent of acid for 30 minutes. Considerable destruction of this substance resulted on boiling it with a concentration of 20 per cent HCl. The determination of glucosane in the earlier experiments was carried out by hydrolysis with 2.5 per cent HCl. Since this was found later to give too low a figure both for glucosane ingested and for that excreted, a correction factor has been applied in those experiments. The results ob-

TABLE I.

Degree of Hydrolysis of Glucosane by Boiling with Water and Dilute Acids.

Concentration of HCl.	Time of boiling.	No. of determinations.	Glucose.			Glucosane.	
			Free.		Total.		
6.0552 gm. salabrose in 1000 cc.							
per cent	*min.*		*gm. in 1000 cc.*	*per cent*	*gm.*	*gm. in 1000 cc.*	*per cent*
0	0	2	0.420	6.93			
0	15	2			0.402	0	0
0	30	2			0.422	0	0
10.000 gm. salabrose in 1000 cc.							
0	0	6	0.739	7.39			
1.25	15	4			6.210	4.924	49.24
2.50	15	12			8.389	6.877	68.77
3.75	15	2			9.484	7.886	78.86
5.00	15	10			9.908	8.242	82.42
5.00	30	2			10.152	8.443	84.43
7.50	15	8			9.859	8.210	82.10
10.00	15	2			9.752	8.127	81.27
20.00	15	2			5.642	4.425	44.25

tained in the later tests, in which the hydrolysis was carried out with 5 per cent HCl, were in agreement with the earlier experiments. Glucosane was calculated by subtraction of the free glucose from the total glucose obtained after hydrolysis. This figure, which represents the glucosane as glucose, is changed to that of glucosane as such by the subtraction of 10 per cent of this amount.

That such a procedure is satisfactory in the determination of the glucosane present in diabetic urine, was shown by the fact

that urine containing glucose, boiled with HCl, remained unchanged in glucose content, while after the addition of glucosane to such a urine subsequent hydrolysis yielded quantitative results for glucosane. The glucosane present in feces was also determined after maceration of the feces in a mortar with water to dissolve the readily soluble glucosane and the determination of the glucose originating after the hydrolysis of an aliquot portion of the filtrate.

The composition of the salabrose solution which we have used was found to be as follows:

	per cent
Glucosane	84.43
Free glucose	7.58
Water	4.40
Total	96.41

The 4 per cent unaccounted for was largely composed of an insoluble residue of carbon. The composition of the glucosane preparations made in this laboratory could likewise be largely accounted for by our experimental procedure. For example, a quantitative analysis of 85 gm. of a glucosane preparation showed the following:

	gm.		*per cent*
Free glucose	40.70	or	47.8
Glucosane	40.82	"	48.0
Total	81.52	"	95.9

The calorimeter experiments were carried out with the respiration calorimeter in the Cornell University Medical College with the usual technique. In the present experiments a water-tight tray,[1] covered with paraffin oil has been arranged under the bed of wire netting on which the dog sleeps in the calorimeter. With this arrangement urine may be collected quantitatively and no appreciable increase in the moisture content of the calorimeter results after voiding, thus eliminating a factor often causing unreliable respiratory quotients. Although this arrangement is unnecessary with well trained calorimeter dogs, it is usually

[1] This procedure was carried out at the kind suggestion of Dr. Zoltán Aszódi, of the University of Budapest.

needed if more than two successive hourly periods are made with diabetic animals on account of their inability to retain in the bladder the increased volume of urine caused by the polyuria.

Results.

Effect of Glucosane on the Respiratory Metabolism of Dogs.—Glucosane, if oxidized, should have a respiratory quotient the same as that of glucose; namely, unity. The following formula indicates the course of this change:

$$C_6H_{10}O_5 + 6\ O_2 \rightarrow 6\ CO_2 + 5\ H_2O.$$

TABLE II.

Metabolism of Dog 163 before and after the Ingestion of Salabrose in Phlorhizin Diabetes. Weight, 8.2 Kilos.

Hourly period ending.	Nature of experiment.	CO_2	O_2	R. Q.	Urinary nitrogen.	Non-protein R. Q.	Total calories.
		gm.	*gm.*		*gm.*		
10.58 a.m.	Basal metabolism,	4.08	4.08	0.727	0.296	0.773	13.60
11.58 "	11th day of phlorhizin.	4.45	4.63	0.699	0.296	0.725	14.84
Averages.....		4.27	4.36	0.712	0.296	0.749	14.22
1.46 p.m.	After ingesting 30	4.68	4.61	0.738	0.248	0.771	15.65
2.46 "	gm. of salabrose at	4.57	4.57	0.727	0.248	0.757	15.25
4.46 "	12.14 p.m.	9.80*	10.01*	0.712*	0.496*	0.734*	32.71*
Averages.....		4.76	4.80	0.722	0.248	0.748	15.61

*2 hour period.

If oxidation of this substance occurs in the diabetic following its ingestion, it should be demonstrated by an increase in the respiratory quotient of a completely phlorhizinized dog, in which the value of the basal respiratory quotient is about 0.70, or of a normal dog after the glycogen has been largely depleted by fasting and the respiratory quotient approaches that of fat (R.Q. = 0.707).

Table II indicates the results obtained after giving 30 gm. of salabrose to a phlorhizinized dog, the basal respiratory quotient of which had been previously determined.

After the administration of 30 gm. of salabrose (25.4 gm. of glu-

cosane) to a phlorhizinized dog weighing 8 kilos there was no rise in respiratory quotient, the average non-protein respiratory quotient for 2 hours before administration being 0.749 and for 4 hours thereafter 0.748. The same results had been obtained in an earlier experiment with Dog 163, as well as in two other animals,

TABLE III.

Metabolism of Dog 162 before and after the Ingestion of Salabrose and of Glucose during Fasting. Weight, 5.70 Kilos.

Hourly period ending.	Nature of experiment.	CO_2	O_2	R. Q.	Urinary nitrogen.	Non-protein R. Q.	Total calories.
		gm.	gm.		gm.		
11.03 a.m.	Basal metabolism,	3.53	3.30	0.778	0.059	0.774	10.90
12.03 p.m.	10th day of fasting.*	3.79	3.81	0.723	0.059	0.712	12.43
Averages		3.66	3.56	0.749	0.059	0.744	11.67
1.46 p.m.	30 gm. of salabrose by	3.75	3.68	0.741	0.080	0.727	12.01
2.46 "	stomach tube at 12.18 p.m.	4.01	3.90	0.748	0.080	0.736	12.76
Averages		3.88	3.79	0.744	0.080	0.732	12.39
12.09 p.m.†	Basal metabolism, 12th day of fasting.	7.36†	7.81†	0.685†	0.134†	0.665†	25.44†
Averages		3.68	3.90	0.685	0.067	0.665	12.72
1.55 p.m.	30 gm. of glucose by	4.39	3.91	0.817	0.094	0.818	13.02
2.55 "	mouth at 12.25 p.m.‡	4.58	4.01	0.831	0.094	0.836	13.41
3.55 "		4.52	4.34	0.757	0.094	0.746	14.25
4.55 "		4.90	5.01	0.710	0.094	0.693	16.33
Averages		4.60	4.32	0.779	0.094	0.773	14.25

* Dog had 250 gm. of meat on 4th day of fast.

† 2 hour period.

‡ Dog excreted 2.9 gm. of glucose in the urine.

data which are given in the protocols at the end of this paper. In only one of these cases was any rise in respiratory quotient noted—in the case of Dog 164 when the respiratory quotient of 0.760 was obtained for 1 hour.

In Table III are given the results of administering 30 gm. of salabrose to Dog 162, a normal fasting animal, as compared to

the results obtained later by the feeding of a like quantity of glucose.

It would appear, not only that it is questionable whether a diabetic dog is able to oxidize glucosane when ingested orally, but likewise that it cannot be demonstrated that a normal animal possesses this power. When 30 gm. of glucose are administered, the rise in respiratory quotient is unmistakable and lasts for 3 hours. One might naturally expect an even higher respiratory quotient after glucosane, were the latter capable of being oxidized, since glucose is so rapidly removed by the liver and muscles to form glycogen, while so far as known there exists no polymer of

TABLE IV.
Alcohol Checks.

Date.	Experiment No.	No. of hours.	Average quotient.	Heat.	
				Calculated.	Found.
1925				*cals. per hr.*	*cals. per hr.*
Dec. 7	200	3	0.668	31.88	31.82
" 8	201	4	0.671	19.69	18.28
" 9	202	4	0.666	21.02	20.24
" 17	203	2	0.668	18.62	18.82
1926					
Jan. 11	205	2	0.669	16.60	14.88
" 25	206	4	0.659	21.15	19.45
" 26	207	5	0.668	16.70	17.32
Averages.....		24	0.667	20.81	20.05

glucosane in the body. According to some of the German investigators (4, 5, 6), glucosane owes its value to the greater ease with which it forms glycogen in the diabetic. If this be true, one should expect a prolonged high D:N ratio in phlorhizinized dogs following glucosane ingestion, a postulate not justified by experiments to be reported later.

The validity of the results obtained with the calorimeter are proved by the alcohol checks for the period in which the experiments reported here were carried out. These are given in Table IV.

The average respiratory quotient for the various alcohol checks was 0.667 (0.6668), which is the theoretical, while the average

heat measured was 20.05 calories, compared with a calculated value of 20.81 calories, a difference of 3.8 per cent.

TABLE V.

Effect of Oral Administration of 30 Gm. of Salabrose on the D:N Ratio of Dog 163. Weight, 7.60 Kilos.

Length of period.	Dextrose.	Nitrogen.		D:N	Glucosane.	Remarks.
		Per period.	Per hr.			
hrs.	*gm.*	*gm.*	*gm.*		*gm.*	
20	21.24	6.416	0.321	3.31		8th day of phlorhizin.
5	5.96	1.555	0.311	3.83	0.649	30 gm. of salabrose, by stomach tube, containing 2.22 gm. of glucose.
6	5.71	1.479	0.247	3.86	0.337	
13	13.58	3.875	0.298	3.50	0.416 0*	
24 hr. total.....					1.402	

* Catheter sample.

TABLE VI.

Effect of Oral Administration of 11.10 Gm. of Glucosane on the D:N Ratio of Dog 160. Weight, 17 Kilos.

Length of period.	Dextrose.	Nitrogen.		D:N	Glucosane.	Remarks.
		Per period.	Per hr.			
hrs.	*gm.*	*gm.*	*gm.*		*gm.*	
18.0	42.912	11.358	0.631	3.80 3.60*		
1.5	4.395	1.076	0.717	4.08	0.108	11.10 gm. of glucosane fed, containing 1.99 gm. of glucose.
1.5	3.390	1.048	0.699	3.23	0.837	
3.0	7.115	2.104	0.701	3.38	0.324	
3.0	7.415	2.031	0.677	3.65	0.108	
3.0	7.136	1.839	0.613	3.88	0.018	
12 hr. total.....					1.395	

* D:N of catheter sample determined separately.

Experiments on the Excretion of Glucose and Glucosane in the Urine after Oral Administration of Glucosane.—After the oral administration of glucosane solutions there is usually an excretion

of this substance in small amounts in the urine, there being no direct quantitative relation between the amount of glucosane administered and that excreted as such in the urine. Representative results of the effect of the ingestion of 30 gm. of salabrose and

TABLE VII.

Effect of Oral Administration of 12 Gm. of Glucose to a Completely Phlorhizinized, Fasting Dog. Dog 160. Weight, 16.7 kilos.

Date.	Length of period.	Dextrose.	Nitrogen.	D:N	Extra-sugar.	Remarks.
1925	*hrs*	*gm.*	*gm.*		*gm.*	
Dec. 13		52.08	15.081	3.45 3.39*		
" 14	24	55.41	12.099	4.50	11.97	12 gm. of glucose by mouth.
		1.66†	0.438†	3.79†		Average ratio = 3.59.

* Catheter sample separately.

† Catheter sample.

Recovery of extra urinary sugar $= \frac{11.97}{12.00} = 99.8$ per cent.

TABLE VIII.

Effect of Oral Administration of 17.22 Gm. of Glucosane, Containing 15.55 Gm. of Glucose, on the D:N Ratio of Dog 162. Weight, 6.20 Kilos.

Length of period.	Dextrose.	Nitrogen.		D:N	Glucosane.	Remarks.
		Per period.	Per hr.			
hrs.	*gm.*	*gm.*	*gm.*		*gm.*	
3	2.025	0.554	0.185	3.65	0	
5	8.401	0.763	0.153	11.01	0.553	35 gm. of glucosane No. 2 given in solution = 15.55 gm. glucose, 17.22 " glucosane.
5	4.633	0.619	0.124	7.48	0.438	
12	8.597	2.430	0.203	3.54	0.127	
					1.118	

of 11.10 gm. of a glucosane preparation on the urinary excretion are given in Tables V and VI. Table VII shows that complete recovery of extra glucose under these conditions could be expected were glucosane converted to glucose or glycogen. Additional experiments are recorded in the protocols at the end of the paper.

It is evident from these experiments that glucosane is not converted into glucose, for if this were the case it would be excreted in the urine as extra sugar, a condition which would result in a greatly increased D:N ratio for the period after its administration. The small increase in D:N ratio after the feeding of glucosane in the experiments given in Tables V and VI can be entirely explained as caused by the elimination of the glucose ingested in the glucosane preparation. Taking the prevailing D:N ratio in Table V as 3.40, the extra sugar excreted amounts to 1.37 gm., as compared with 2.22 gm. of glucose fed. Were glucose absorbed as formed from the glucosane under the conditions of this experiment, it would be excreted as extra sugar in the urine, as evidenced by the results tabulated in Table VII. When 12 gm. of glucose were given to Dog 160 there was an excretion of extra glucose in the urine of 11.97 gm., or 99.8 per cent of that ingested.[2]

When glucosane preparations containing large amounts of unconverted glucose are fed to phlorhizinized dogs, there is a marked rise in D:N ratio—a rise, however, which can be accounted for entirely as a result of ingested glucose. Table VIII shows the results when 17.22 gm. of glucosane, containing 15.55 gm. of glucose, were fed to Dog 162.

There is another postulate as to the ultimate fate of the glucosane; namely, that it may act as a glycogen former in the diabetic more satisfactorily than may glucose. Nothmann and Kühnau (6), on the basis of several experiments on fasting rabbits to which glucosane was fed, conclude that it may serve as a glycogenetic agent inasmuch as the glycogen content of the livers of these animals was higher than that after a like period of fasting. However, it is well to remember that rabbits are herbivora, with the processes of digestion considerably different from those of man. These investigators also claim an increased glycogen content in the livers of depancreatized dogs after glucosane feeding over that normally found under such conditions, although they base their conclusions on only a 1 per cent concentration of glycogen in the liver.

In the experiments reported here there is no evidence that in

[2] Extra glucose is calculated by considering the prevailing D:N ratio during the day of the experiment as the average of that before and after, 3.39 and 3.79, or an average of 3.59.

the phlorhizinized dogs a conversion of glucosane to glycogen occurs. Were this the case, we should expect an increased D:N ratio both during the day in which the material was fed and possibly for a 2nd day or more thereafter. When large stores of glycogen are present in the liver during the 1st and 2nd days of phlorhizin glycosuria, there is usually a high glucose elimination coupled with a high D:N ratio, which has been interpreted to indicate the flushing out of the bulk of the glycogen reservoirs. In the four experiments reported here the D:N ratios on the days following glucosane administration were 3.54, 3.88, 3.50, and 3.65, values which preclude the possibility of any large reservoirs of glycogen being broken down. Likewise, on the days of glucosane ingestion, when this was not accompanied by the administration of a large amount of glucose, the usual fasting D:N ratios of 3.65 were only slightly increased, as given in Tables V and VI.

Experiments on the Absorption of Glucosane.—Since it could not be proved from respiration experiments that glucosane was oxidized in either the diabetic or the normal dog, and since only about 5 to 10 per cent of the ingested glucosane was excreted in the urine as such and no proof of the conversion of the remainder of the glucosane into either glucose or glycogen could be found, it seemed of interest to determine whether it might be largely unabsorbed from the gastrointestinal tract. This seemed a probable explanation, since in some of our earlier respiration experiments in which our own glucosane solutions were fed to the dog it excreted black tarry feces 6 to 8 hours after the administration of glucosane which were readily soluble and appeared, from qualitative tests, to contain a material which reduced copper solutions after hydrolysis with HCl.

A few experiments were carried out on normal dogs fed on a standard diet containing all the essential foodstuffs, such as were used in earlier experiments (15). The presence of bone ash in this diet caused the production of well formed stools which made their quantitative collection accurate. With sucrose the only carbohydrate in the diet, the presence of any other unutilized carbohydrate than glucosane in the feces was avoided.

The analyses of the feces were carried out as described earlier. Table IX summarizes our data on this point.

After giving 30 gm. of salabrose, which contained 25.33 gm.

of glucosane, to Dog 162, 17.99 gm., or 71 per cent, were recovered in the feces. A considerable amount of glucosane was excreted as late as the 7th day after its oral administration. Total glucose after hydrolysis was considered in these experiments as having

TABLE IX.

Elimination of Glucosane in the Feces Following the Ingestion of 30 Gm. of Salabrose by Dog 162. Weight, 6.2 Kilos.

Date.	Day after glucosane feeding.	Glucosane.	Remarks.
1926		*gm.*	
Jan. 27	0	0.42*	30 gm. of salabrose by stomach tube.
" 28	1	9.13	
" 30	3	4.80	
" 31	4	2.66	
Feb. 1	5	0.24	
" 3	7	1.16	
" 4	8		Feces discarded.
" 5	9		" "
" 6	10	0.07	Control period.
" 8	12	0.05	
Total in 7 days.....		17.99	

* Urine for 4½ hours after glucosane only; not included in total.

TABLE X.

Recovery of Glucosane in the Feces after Its Oral Administration.

Animal.	Glucosane.		
	Fed.	Recovered.	
	gm.	*gm.*	*per cent*
Dog 162	25.33	17.99	71
" 163	11.51	5.80	50
" 163	25.33*	11.65*	
" 165	25.33	11.15	44

* Only one defecation 12 hours after administration.

been derived from glucosane since no reducing sugars are excreted in the feces, as is demonstrated in the control period when small amounts of glucosane were still being excreted.

In other experiments considerable amounts of glucosane were

found in the feces, but not as large a recovery was obtained. The summarized results are given in Table X.

There is a large portion of the ingested glucosane which is unabsorbed and eliminated in the feces. The excretion takes place slowly over a considerable period of time, large amounts of glucosane being present in the stools 10 days after its administration in the case of Dog 164.

Just what happens to the rest of the glucosane is problematic. As much as 70 per cent of the ingested glucosane was recovered in the feces. It is possible that the remaining part unaccounted for is slowly excreted in the urine and possibly in the feces in amounts too small to determine. It may be also in part destroyed by some of the intestinal bacteria, although it is not fermentable by yeast nor acted on by *Bacillus coli*.

Experiments on the Subcutaneous and Intraperitoneal Injection of Glucosane.—In order to determine whether it might be possible to oxidize glucosane in case this substance was absorbed, a salabrose solution was injected subcutaneously and intraperitoneally in normal dogs. Three experiments on two dogs were carried out with subcutaneous injection of 11.52 gm. of glucosane, containing 1.04 gm. of glucose, in sterile saline, and six experiments in which the same amount was injected intraperitoneally in 100 cc. of sterile saline in three different animals. Tables XI, XII, and XIII summarize the data.

After the subcutaneous or intraperitoneal injection of glucosane there was an excretion of large amounts of this substance in the urine almost immediately. It was eliminated at such a rate that some of the urine excreted 4 to 5 hours after the administration of salabrose, which had failed to drain completely from the flat trays of the metabolism cages, turned quickly into a thick, black, syrupy residue resembling our glucosane solution. This mass was readily soluble in water.

After the subcutaneous injection of 13.64 gm. of salabrose into normal dogs there was an excretion of the largest proportion in the urine the 1st day, a much smaller proportion the 2nd day, with a trace the 3rd day. Similarly, there was a considerable edema still present the 2nd day after injection which had practically disappeared on the 3rd day.

When a like amount of salabrose in the same concentration was

TABLE XI.

Recovery of Glucosane in the Urine after the Subcutaneous Injection of 11.55 Gm. into a Normal Dog. Dog 165. Weight, 7.95 Kilos.

Length of time after glucosane.	Glucose.		Glucosane.
	Total.	Free.	
hrs.	*gm.*	*gm.*	*gm.*
25	11.65	0.87	9.70
28	1.12	0.54	0.52
24	0.50	0.31	0.15
Total.....			10.37 or 90 per cent.

TABLE XII.

Recovery of Glucosane in the Urine after the Intraperitoneal Injection of 11.55 Gm. into a Normal Dog. Dog 165. Weight, 7.85 Kilos.

Length of time after glucosane.	Glucose.		Glucosane.
	Total.	Free.	
hrs.	*gm.*	*gm.*	*gm.*
24	12.95	1.37	10.42
24	0.76	0.60	0.14
22	0.57	0.60	0
Total.....			10.56 or 91 per cent.

TABLE XIII.

Recovery of Glucosane in the Urine after Subcutaneous and Intraperitoneal Injection into Normal Dogs.

Animal.	Method of administration.	Glucosane.		
		Injected.	Recovered.	
		gm.	*gm.*	*per cent*
Dog 163	Subcutaneous.	11.55	Positive qualitative.	
" 165	"	11.55	12.03	104
" 165	"	11.55	10.37	90
" 165	Intraperitoneal.	11.55	11.51	100
" 165	"	11.55	9.88	86
" 165	"	11.55	10.56	91
" 168	"	11.43	10.69	94
" 163	"	11.55*	9.23*	80*
" 163	"	11.55	Positive qualitative.	

* Possibly some urine was lost in this experiment.

injected intraperitoneally into normal dogs, the same phenomena were noted, although practically all the glucosane was excreted on the 1st day after its administration. While subcutaneous injection did not result in any noticeable disturbances in our animals, after the intraperitoneal administration the presence of the large amount of fluid in the peritoneal cavity sometimes caused distressing symptoms, such as vomiting, defecation, and nausea, which usually persisted for a day.

There was a practically complete recovery of injected glucosane both after the intraperitoneal administration (100, 86, 91, 94, and 80 per cent) and after the subcutaneous injection (104 and 90 per cent) of 13.64 gm. of salabrose into normal dogs, results which may be interpreted as representing a quantitative elimination. It would seem from these data that the body possessed no catalyst or enzyme which is capable of converting this substance to glucose or glycogen in case it is absorbed nor one for the oxidation of measurable quantities thereof. The experiments in which glucosane was given intraperitoneally and subcutaneously show without exception that glucosane is excreted as such and not as glucose. Were it converted into glucose in the normal animal, we should expect that the glucose so formed would be promptly oxidized.

Although no experiments were carried out in which the glucosane was introduced intravenously, such experiments have been performed by Kerb (16). This investigator concluded that glucosane must be rapidly taken up by the tissues, but that it is only slowly converted to combustible carbohydrate. No analyses were made to determine its presence in the urine. However, there is no reason to believe that its fate is different when introduced by the intravenous pathway from that after its subcutaneous or intraperitoneal administration.

DISCUSSION.

In the experiments reported here no evidence could be found to show that glucosane can be utilized when given to either normal or diabetic (phlorhizin) dogs whether the substance be administered *per os*, intraperitoneally, or subcutaneously. When given *per os* to normal or phlorhizinized dogs there was no increase in the respiratory quotient. The idea that glucosane is convertible into glucose or into glycogen by the organism is likewise un-

founded, for when a glucosane solution (containing only a very little glucose) was fed to a fasting, phlorhizinized dog, the prevailing D:N ratio of 3.65 was scarcely changed on the day of its ingestion or on the day following its administration. Had it been converted into glycogen, it would have been eliminated as extra sugar in the urine, since glycogenolysis readily takes place in the phlorhizinized dog.

The truth of the matter of the fate of glucosane given to a dog appears to be that it is largely unabsorbed, and the portion which is absorbed is not oxidized but is completely eliminated in the urine. For we have found in one normal dog to which it was given that 71 per cent was eliminated in the feces, and also we have always found glucosane in the urine during the first 6 to 8 hours after its ingestion. Furthermore, when glucosane is administered intraperitoneally or subcutaneously it may be almost completely recovered, chiefly in the urine of the first 24 hours and in small part of the next 24 hours, indicating that it is not oxidized or chemically changed by the living organs of the body and is not therefore a physiologically useful product.

These experiments render it improbable that glucosane can be oxidized in human diabetes. It should be remembered, as has recently been aptly expressed, that human diabetics are for the most part only "partially depancreatized animals." Hence, changes in their urinary sugar output are greatly influenced by dietary regulations. The output of sugar is especially reduced by a low protein intake, which reduction is liable to be interpreted in investigations upon human diabetics as being due to an added food factor—glucosane, for example—whereas in reality the benefit is due to an entirely different factor, the beneficent character of the diet.

SUMMARY.

After the administration of 30 gm. of salabrose, a commercial preparation of glucosane, to either phlorhizinized or normal, fasting dogs no increase in the respiratory quotient resulted, indicating that this substance did not undergo oxidation during the period in which the determinations were carried out.

There was no evidence of the conversion of glucosane into either glucose or glycogen, when introduced orally in phlorhizinized

dogs or after subcutaneous or intraperitoneal injection in normal dogs.

Some of the glucosane preparations caused a diarrhea in which large amounts of unchanged glucosane were excreted in the feces. However, even when a diarrhea did not occur after its administration, large amounts were excreted in the feces, appreciable quantities being present at least a week after its administration. As large an amount as 71 per cent was recovered in the period of a week after its ingestion.

After intraperitoneal or subcutaneous injection of this substance in normal dogs there was a practically quantitative recovery of unchanged glucosane in the urine, indicating the inability of the organism to oxidize it, to store it, or to convert it into glucose or glycogen.

A method is described for the quantitative determination of this substance in the urine.

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PROTOCOL 1.

Metabolism of Dog 163 before and after the Ingestion of Salabrose in Phlorhizin Diabetes. Weight, 8.20 Kilos.

Hourly period ending.	Nature of experiment.	CO_2	O_2	R. Q.	Urinary nitrogen.	Non-protein R. Q.	Total calories.
		gm.	gm.		gm.		
12.19 p.m.	Basal, 5th day of	4.76	4.80	0.721	0.305	0.755	15 86
1.19 "	phlorhizin. D:N	5.00	5.17	0.703	0.305	0.727	16.67
2.19 "	= 4.28.	5.05	5.14	0.715	0.305	0.743	16.84
Averages.....		4.94	5.04	0.713	0.305	0.741	16.46
12.18 p.m.*	Basal, 7th day of	10.43*	10.46*	0.724*	0.546*	0.752*	34.82*
1.18 "	phlorhizin. D:N = 3.45.	5.53	5.57	0.722	0.273	0.746	18.46
Averages.....		5.32	5.34	0.723	0.273	0.750	17.76
1.01 p.m.	30 gm. of salabrose	5.02	4.90	0.745	0.311	0.790	16.75
2.01 "	by mouth at 11.15	4.63	4.66	0.723	0.311	0.761	15.44
3.01 "	a.m.; 8th day of	5.04	5.18	0.708	0.311	0.735	16.82
4.01 "	phlorhizin.	4.67	4.74	0.717	0.311	0.751	15.57
Averages.....		4.84	4.87	0.723	0.311	0.759	16.15

*2 hour period.

PROTOCOL 2.

Metabolism of Dog 164 before and after the Ingestion of Salabrose during Phlorhizin Diabetes. Weight, 13.40 Kilos.

Hourly period ending.	Nature of experiment.	CO_2	O_2	R. Q.	Urinary nitrogen.	Non-protein R. Q.	Total calories.
		gm.	gm.		gm.		
1.00 p.m.*	Basal, 5th day of	17.93*	18.82*	0.693*	0.898*	0.708*	59.83*
3.00 "	phlorhizin. D:N = 3.64.	17.58*	18.26*	0.700*	0.898*	0.718*	58.65*
Averages.....		8.88	9.27	0.696	0.449	0.713	29.63
11.03 a.m.	30 gm. of salabrose	7.60	7.27	0.760	0.388	0.800	25.37
12.03 p.m.	by stomach tube	7.65	7.87	0.707	0.388	0.727	25.54
1.03 "	at 9.12 a.m.	7.73	7.83	0.718	0.388	0.742	25.81
2.03 "		7.62	7.72	0.718	0.388	0.742	25.44
Averages.....		7.65	7.67	0.725	0.388	0.752	25.54

*2 hour period.

PROTOCOL 3.

Effect of Oral Administration of Glucosane on Dog 162. Weight 6.20 Kilos.

Length of period.	Dextrose.	Nitrogen.		D:N	Glucosane.	Remarks.
		Per period.	Per hr.			
hrs.	*gm.*	*gm.*	*gm.*		*gm.*	
3	2.526	0.681	0.227	3.71		
5	9.615	0.905	0.181	10.62	0.346	17.22 gm. of glucosane, containing 15.55 gm. of glucose, given by stomach tube.
4	4.780	0.697	0.174	6.86	0.427	
3	2.186	0.557	0.186	3.92	0.094	
3	2.025	0.554	0.185	3.65	0	
15 hr. total.....					0.867	

PROTOCOL 4.

Effect of Oral Administrationof Salabrose on Dog 164. Weight, 13.40 Kilos.

Length of period.	Dextrose.	Nitrogen.	D:N	Glucosane.	Remarks.
hrs.	*gm.*	*gm.*		*gm.*	
5.5	8.98	2.468	3.64		6th day of phlorhizin.
Catheter sample.	14.74	4.234	3.48		7th " " "
5	9.50	1.939	4.90	0.340	30 gm. of salabrose by stomach tube.
24	32.09	8.206	3.91	0	

ANTIRICKETIC SUBSTANCES.

IV. THE POLYMERIZATION OF CHOLESTEROL.

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Plates 1 to 3.

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In a previous communication (1) it was reported that the catalytic action of floridin on cholesterol gives a series of colored, fluorescent, odorous, resinous, and antiricketic substances. Considering the work of Gurvich (2), Venable (3), and Lebedev and Filonenko (4) this reaction, in a general way, was predictable for an olefinic terpene such as cholesterol. We believe it affords a valuable method for investigating the sterols, which recently have been associated with the fat-soluble vitamins.

To perform the catalysis our usual procedure has been to reflux a carbon tetrachloride solution of cholesterol with activated floridin, the relative proportions being 30 ml. of solvent, 1 gm. of cholesterol, and 5 gm. of floridin. The progress of the reaction is indicated by a series of color changes in the catalyst: old rose → reddish purple → bluish purple → purplish black → black (1).

We have now isolated an early product of the catalysis, which we tentatively term "tricholesterol." This is the precursor of the later colored and antiricketic substances. Separation was accomplished by precipitating the tricholesterol out of its carbon tetrachloride solution by adding alcohol, in which it is exceedingly insoluble. To prepare the material the catalytic action must be moderated by hindering in various ways the latter stages thereof, and thus preventing in a large measure the formation of colored decomposition products.

1. By Employing Unactivated Floridin.—The floridin as received contained 19.1 per cent of water expellable at 880°. Or-

dinarily, when we speak of activated floridin, we refer to material having a water content of 5 to 4 per cent (300°), and exhibiting maximum activity. The unactivated floridin when refluxed for 3.5 hours gave a small yield of tricholesterol containing a persistent impurity.

2. By Impairing the Activity of the Catalyst.—As floridin is dehydrated its activity gradually increases to the maximum and then rapidly drops, the completely dehydrated material being practically inactive. However, some floridin containing as little as 0.1 per cent of water (700°) gave a good yield of tricholesterol after 2 hours refluxing.

3. By Employing a Lower Temperature for Catalysis.—A preparation containing activated floridin was incubated with occasional shaking at 37.5°. In 4 days it gave a good yield, whereas a similar preparation at 5° gave no reaction in a month.

4. By Abbreviating the Period of Refluxing.—When a preparation with activated floridin was refluxed for a much shorter time than is required to demonstrate the entire series of color changes, a good yield was easily obtained. This being our best procedure, it is given in more detail.

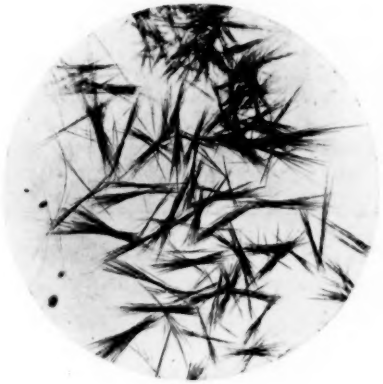
Reflux in an 800 ml. Kjeldahl flask 10 gm. of cholesterol plus 300 ml. of carbon tetrachloride plus 50 gm. of activated floridin until the suspension assumes a reddish purple color (not bluish purple). Approximately 15 minutes will be required, during which agitate the flask constantly to prevent the sediment from overheating or drying on the walls. Filter with suction, lixiviate with carbon tetrachloride, and reduce the combined filtrate and lixivium to 300 ml. The solution should be colorless if the refluxing has not been carried too far. Add 300 ml. of 95 per cent ethyl alcohol. A voluminous amorphous precipitate should appear at once. Heat the solution until the precipitate suddenly turns crystalline, then allow to cool. Filter, and wash with a freshly prepared mixture[1] of one part tetrachloride and two parts alcohol by volume. Press out, and dry at 100°. Recrystallize by dissolving in 150 ml. of tetrachloride, and adding 150 ml. of alcohol. Heat, cool, filter, wash, and dry as before. The yield of about 40 per cent may be increased by working up the filtrates.

By cooling a hot solution of tricholesterol in equal volumes of carbon tetrachloride and alcohol it is obtained in fine, colorless, needles barely visible to the naked eye (Text-figs. 1 and 2). If the

[1] This mixture on standing several days becomes strongly acid, presumably through the formation of phosgene or other chlorinated compounds.

hot solution is very concentrated the crystals may separate as a haystack gel. If the concentrated solution is somewhat impure from pigmented substances, or if an excess of alcohol is added,

TEXT–FIG. 1.



TEXT–FIG. 2.

TEXT-FIGS. 1 and 2. Crystal forms of tricholesterol. × about 65.

the tricholesterol may be thrown out in the amorphous form. A characteristic of the pure substance is that when it is precipitated from tetrachloride in the amorphous condition by the rapid addi-

tion of an equal volume of alcohol, this precipitate suddenly becomes crystalline when the solution is warmed.

The solubilities of tricholesterol are recorded in Table I.

Like several other cholesterol derivatives, tricholesterol exhibits a multiple melting point. At 203° it melts without decomposition to a crystalline liquid which becomes clear at 209°. When this liquid is cooled to the solidification point, the resulting crystalline mass remelts at 196°, giving the doubly refracting liquid which again becomes clear at 209°.

In optical activity tricholesterol is levorotatory. For a 2 per cent solution in carbon tetrachloride $[\alpha]_D^{20} = -40.8°$; for cholesterol under the same conditions $[\alpha]_D^{20} = -38.4°$.

TABLE I.

Showing to Two Significant Figures the Number of Mg. of Tricholesterol in 100 Ml. of Solution Saturated at 25°.

Solvent.	Solubility.
	mg.
Carbon tetrachloride	13,000
Chloroform	5,800
Benzene	3,400
Toluene	2,800
Ethyl ether (absolute)	460
" acetate	45
Isobutyl alcohol	33
Ethyl alcohol (absolute)	3
Acetone	3
Water	0

The chemical nature of tricholesterol was deduced from its empirical analysis and molecular weight.

Percentage Composition.

	C	H	O (by difference).
Theoretical for any polymer of cholesterol...	83.9	12.0	4.1
Found for 0.2 gm. sample of X.....	83.6	11.7	4.7

Although the probable analytical error would cover a variation of a few atoms of H, the mode of preparing the polymer (catalysis) makes it most unlikely that any gain or loss of H took place.

Determining the melting point depression of a 6 per cent solu-

tion of tricholesterol in *d*-camphor by the method of Rast (5), the computation for the molecular weight was 978, the theoretical value being 1159. The observed value is thus 15.6 per cent short of the theoretical, but it is 26.5 per cent greater than the theoretical value for the hypothetical dicholesterol. By the same technique the observed molecular weight of purified cholesterol was 5.9 per cent short of the theoretical; therefore, the direction of the error in this case supports the assumption that the new compound is a trimer of cholesterol. While experimental errors of this magnitude are not above suspicion, we do not consider them extraordinary for compounds of such high molecular weight.

In the polymerization of cholesterol something happens to the alcohol group, for the polymer cannot be acetylated under the following conditions. 0.5089 gm. of tricholesterol in 5 ml. of xylene was refluxed for 30 minutes with 10 ml. of acetic anhydride. The product after evaporation to dryness was slightly colored and it weighed 0.5097 gm., which indicates that no significant acetylation took place. Under the same conditions cholesterol was acetylated almost quantitatively.

Tricholesterol gives the Salkowski and Burchard-Liebermann color reactions the same as cholesterol, except for momentary differences in the first play of color. Moleschott's reaction, however, is given only feebly, especially so when the sulfuric acid is dilute.

Tricholesterol absorbs iodine from Hubl's solution somewhat less readily than cholesterol. The quantity absorbed varies widely with the time and temperature, so that it is impossible to ascribe a definite iodine value to tricholesterol by Hubl's method. In our experience the same is true of cholesterol, for which Lewkowitsch (6) reports a Hubl value close to the theoretical.

Tricholesterol is not antiricketic, and, unlike cholesterol, it does not become so by exposure to ultra-violet rays. Some tricholesterol was dissolved in ether, taken up in seal oil, and triturated with McCollum's Diet 3143 in such proportions that the modified diet contained 0.5 per cent of tricholesterol and 2 per cent of seal oil. This preparation induced no healing in rickety rats. A similar preparation was made with tricholesterol which had been irradiated for 15 minutes under a Cooper Hewitt "Uviarc" operating at 150 volts. The burner was 36 cm. above the tricholesterol,

TABLE II.

Protocols of Rats.

Rat No.	Days on test.	Preparation administered.	Grade of test.	Weight.	Average daily consumption.
				gm.	gm.
1709	5	Control: Diet 3143 plus seal oil 2 per cent.	–	99–99	7.8
1725	5	" " "	–	75–79	7.6
1746	5	" " "	–	59–60	5.4
1753	5	" " "	–	81–87	7.8
1772	5	" " "	–	82–86	7.2
1733	5	Tricholesterol ½ per cent plus seal oil 2 per cent.	–	71–71	6.6
1734	5	" " "	–	71–72	7.2
1735	5	" " "	–	72–72	5.2
1736	5	" " "	–	70–72	8.0
1762	5	" " "	–	67–67	6.6
1713	5	Irradiated tricholesterol $\frac{1}{10}$ per cent plus seal oil 2 per cent.	–	77–79	6.8
1714	5	" " "	–	95–98	7.2
1750	5	Irradiated tricholesterol ½ per cent plus seal oil 2 per cent.	–	95–96	8.8
1751	5	" " "	–	74–76	7.6
1723	5	Catalyzed tricholesterol ½ per cent plus seal oil 2 per cent.	++	94–96	9.0
1738	5	" " "	++	75–75	6.6
1742	5	" " "	+++	73–73	6.6
1759	5	" " "	++	79–79	6.6
1760	5	" " "	++	78–80	7.0
1814	7	Catalyzed tricholesterol $\frac{1}{10}$ per cent plus seal oil 2 per cent.	–	67–70	6.0
1816	7	" " "	+	65–73	8.3
1817	7	" " "	– (?)	72–72	6.4
1818	7	" " "	+	78–84	7.3
1819	7	" " "	– (?)	66–70	8.0
1820	7	Catalyzed tricholesterol 2 per cent plus seal oil 2 per cent.	++++	63–59	5.4
1821	7	" " "	++++	84–79	6.1
1823	7	" " "	++++	75–68	4.7
1824	7	" " "	++++	83–79	5.4
1825	7	" " "	++++	86–80	6.9

TABLE II—*Concluded.*

Rat No.	Days on test.	Preparation administered.	Grade of test.	Weight.	Average daily consumption.
				gm.	*gm.*
1853	5	Catalyzed tricholesterol $\frac{1}{2}$ per cent, treated with *n*-butyl nitrite, plus seal oil 2 per cent.	+	64–68	5.6
1854	5	" " "	++	69–71	6.6
1855	5	" " "	++	65–70	6.6
1856	5	" " "	++	66–68	6.8
1857	5	" " "	−	67–67	5.2
1808	7	Catalyzed tricholesterol $\frac{1}{10}$ per cent, treated with *n*-butyl nitrite, plus seal oil 2 per cent.	−	68–70	6.9
1809	7	" " "	−	82–91	7.6
1810	7	" " "	+	61–66	6.1
1811	7	" " "	−	80–80	6.9
1812	7	" " "	− (?)	67–72	7.3

which was exposed as a very shallow layer of fine powder. It has been previously shown (7) that 0.1 per cent of cholesterol irradiated in exactly this manner exhibits a distinct healing action in rickets; the irradiated tricholesterol, on the contrary, gave no action, although the dose administered was five times as large (Table II).

The polymerization of olefinic terpenes and other olefines by floridin has been studied by several investigators. Gurvich (2, 8) discusses the phenomena observed in the polymerization of pinene. He concludes that there occurs first a polymerization, followed by depolymerization resulting from the heat of reaction. On the other hand, Venable (3), after investigating several terpenes, concludes that the initial change is an intramolecular rearrangement, followed by polymerization, and, at elevated temperatures, by a degradation of the polymers into hydrocarbons. Lebedev and Filonenko (4) mention briefly that the polymers of various double bond compounds depolymerize when warmed with floridin.

We find that purified tricholesterol is degraded by further treatment with tetrachloride and floridin. The decomposition in this

case is obviously catalytic and not caused by the temperature *per se*, since tricholesterol is perfectly stable at the boiling point of tetrachloride. Some tricholesterol was dissolved in carbon tetrachloride and refluxed for 5 hours with activated floridin, the proportions being the same as in all previous experiments with cholesterol. The color changes in the catalyst were the same as for cholesterol, except that they commenced immediately upon warming. The suspension was filtered, and the floridin residue extracted with ether and acetone until no more coloring passed into solution. The combined filtrate and extracts exhibited a deep orange color with a slight fluorescence. To all appearances this product is the same as the antiricketic resin (catalyzed cholesterol) obtained directly from cholesterol by the prolonged action of floridin (1).

The catalyzed tricholesterol thus obtained exhibited approximately the same antiricketic action as the catalyzed cholesterol previously described. After gentle evaporation of the solvents, it was incorporated with seal oil and Diet 3143 in such proportions that the modified diet contained 0.5 per cent of catalyzed tricholesterol and 2 per cent of seal oil. This preparation induced advanced healing in 5 days. However, 0.1 per cent of catalyzed tricholesterol induced only faint healing in 7 days. A dose of 2.0 per cent promoted an extremely heavy deposit of calcium in 7 days, but this was accompanied by a loss of weight in the rats.

In a previous communication (7) it was demonstrated that both the antiricketic vitamin and irradiated cholesterol are inactivated by *n*-butyl nitrite under certain conditions. This reaction was applied to the new product by dissolving 1.5 gm. of catalyzed tricholesterol in 6 gm. of seal oil, and adding 3 ml. of *n*-butyl nitrite. The mixture was placed in a small test-tube plugged with cotton and immersed in boiling water for 1 hour. The product was made up to 300 gm. with Diet 3143, so that the modified diet contained 0.5 per cent of treated catalyzed tricholesterol and 2 per cent of seal oil. Before administration it was exposed to air to dissipate the odor. The tests with rats indicated that no significant diminution in antiricketic action occurred. A similar preparation containing only 0.1 per cent of treated catalyzed tricholesterol gave very feeble healing, the same as that given by catalyzed tricholesterol before treatment with nitrite.

The nature of the antiricketic substance in catalyzed cholesterol (or catalyzed tricholesterol) therefore remains obscure. The nitrite reaction proves it is not identical with irradiated cholesterol or the antiricketic vitamin. From the fact that large doses (2 per cent) of catalyzed tricholesterol are accompanied by a marked loss of weight but normal food consumption, the healing action may be interpreted in either of two ways. The first possibility is that it results from a systemic disturbance following mild poisoning by the resinous mixture. Occasionally, among large numbers of rickety rats receiving cod liver oil, etc., we find a rat which loses weight during the experimental period for no apparent cause. It is our custom to discard the records of such cases, because they are often untrustworthy, especially if healing is indicated. But in the present case all the rats receiving 2 per cent of catalyzed tricholesterol lost weight; this can hardly be fortuitous, and hence the records are included in Table II as typical of this preparation. The second possibility is that there is present in catalyzed tricholesterol a non-toxic antiricketic substance, responsible for the healing at 0.5 per cent where the rats gained weight. Then in addition to this there may be mildly toxic substances present which cause the loss of weight at 2.0 per cent. The situation at present reminds us of the healing observed by Shipley, Kinney, and McCollum (9) in rats receiving oil of cloves. The protocols of all the rats are combined in Table II, and the typical degrees of healing, from severe rickets to advanced calcification, are illustrated in Figs. 1 to 6 of Plates 1 to 3.

As there is a growing tendency to associate vitamin A, as well as D, with cholesterol, we ventured to treat some rats exhibiting incipient dietary ophthalmia with catalyzed cholesterol. A dose of 0.5 per cent plus 2 per cent of olive oil was incorporated in the diet and administered to two rats. These rats showed no improvement in 10 days, whereas two control rats receiving 1.0 per cent of cod liver oil were cured in this period.

There is a suggestion resulting from our experiments, that irradiated cholesterol and the natural vitamin D may be polymeric forms of cholesterol. We have demonstrated the fact that cholesterol is polymerizable, albeit tricholesterol is not antiricketic. The molecular structures on which catalytic polymerization occurs may, in analogous compounds, be polymerized by ultra-violet rays.

SUMMARY.

1. Cholesterol on treatment with activated floridin behaves in general as an olefinic terpene. It is first polymerized, and the polymer is then degraded by further catalytic action into a resinous mass.

2. The polymer appears to be tricholesterol, $C_{81}H_{138}O_3$; molecular weight, theoretical, 1159, found, 978; $[\alpha]_D^{20} = -40.8°$ (2 per cent in CCl_4); crystalline form, microscopic needles from CCl_4—C_2H_5OH; principal melting point 203°; secondary melting points 209° and 196°; solubilities as described.

3. Tricholesterol gives the Burchard-Liebermann, Salkowski, and Moleschott cholesterol color reactions with slight differences; it absorbs iodine erratically from Hubl's solution; it is not acetylated on treatment with acetic anhydride.

4. Tricholesterol is not antiricketic and does not become so by irradiation. However, on further treatment with floridin it gives an antiricketic degradation product which apparently is identical with the product obtained directly from cholesterol. The antiricketic substance is different from the antiricketic vitamin and irradiated cholesterol, and its mode of action is not understood.

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EXPLANATION OF PLATES.

The accompanying camera lucida standards of the line test were made by the medical artist, Mr James F. Didusch, in 1924. The dissections were prepared by one of us (C. E. B.), with the kind approval of Dr. P. G. Shipley of the Johns Hopkins Medical School.

PLATE 1.

FIG. 1. Normal tibia. Tibia from a young rat which never had rickets.
FIG. 2. Severe rickets. Extreme rickets produced by Diet 3143.

PLATE 2.

FIG. 3. + Healing rickets, indicating a just perceptible healing.
FIG. 4. ++ Healing rickets, indicating a distinct healing.

PLATE 3.

FIG. 5. +++ Healing rickets, indicating an advanced healing.
FIG. 6. ++++ Healing rickets, indicating a practically complete healing, the amount of calcium being normal, and only some structural evidences of rickets remaining.

b.t. = bony trabecula.
cart. = cartilage.
cort. = cortex.
dia. = diaphysis.
ep. = epiphysis.
ep.d.cart. = epiphysial disc of cartilage.
mar. = marrow.
met. = metaphysis.
mus. = muscle, etc.
os. = osteoid.
p.z.c. = provisional zone of calcification.

THE

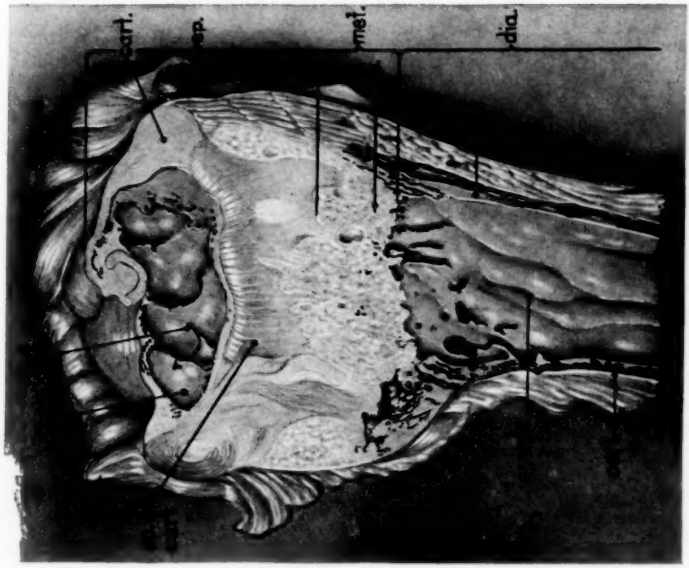

FIG. 2. SEVERE RICKETS ×10

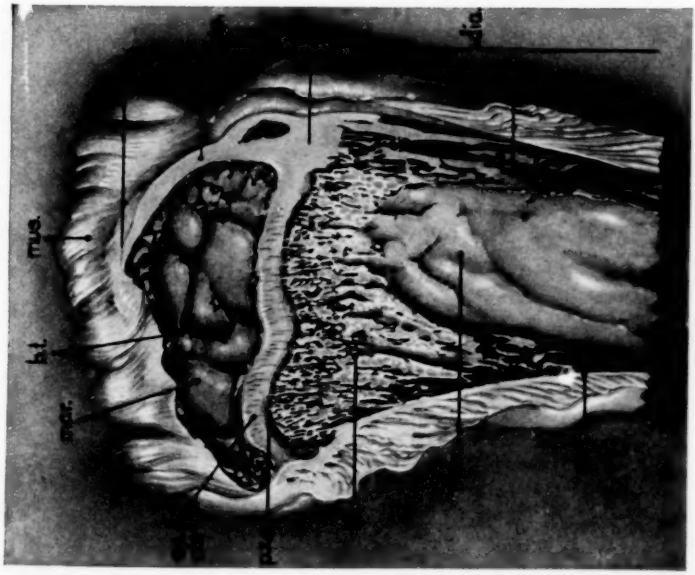

FIG. 1. NORMAL TIBIA ×10

(Bills and McDonald: Antiricketic substances. IV.)

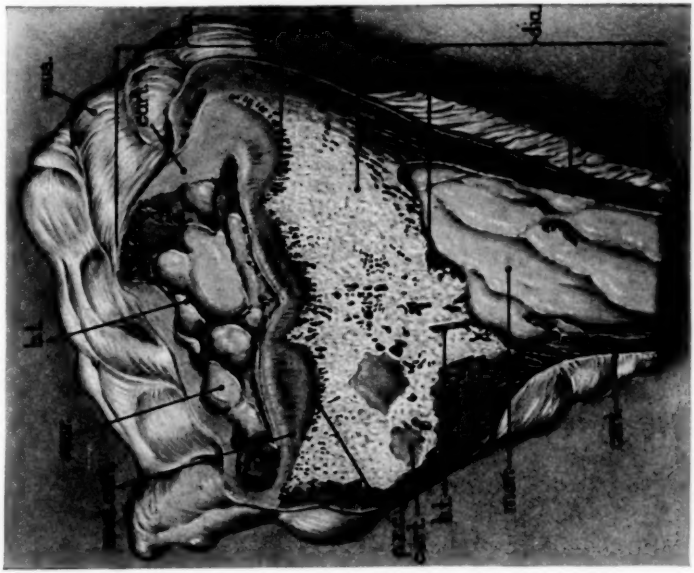

FIG. 4. ++ HEALING RICKETS ×10

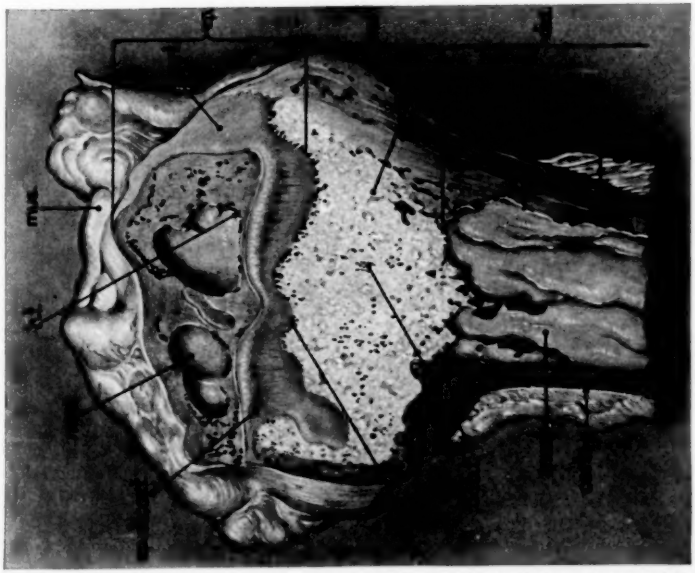

FIG. 3. + HEALING RICKETS ×10

(Bills and McDonald: Antiricketic substances. IV.)

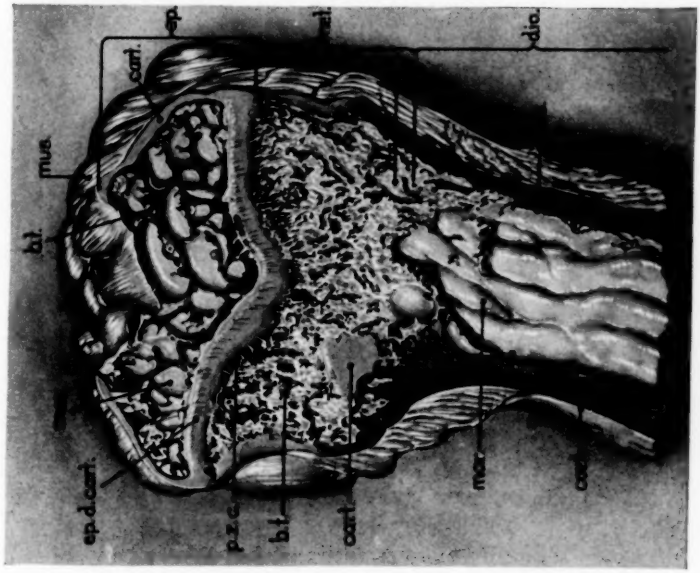

FIG. 6. ++++ HEALING RICKETS ×10

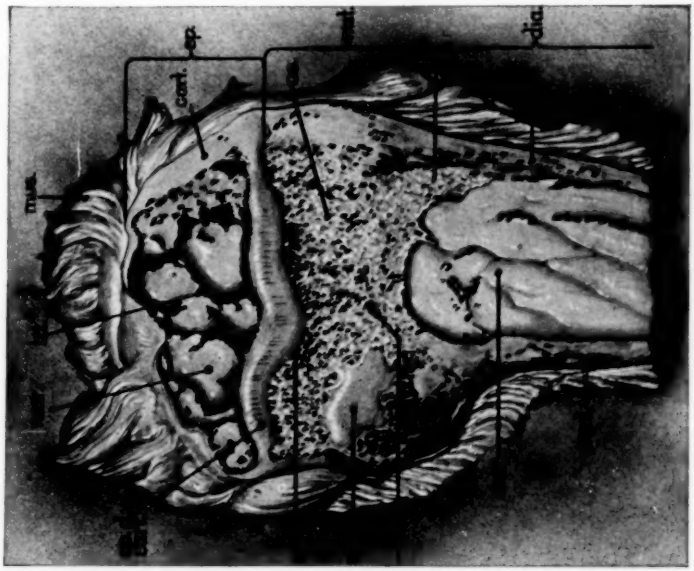

FIG. 5. +++ HEALING RICKETS ×10

(Bills and McDonald: Antiricketic substances. IV.)

THE EFFECT OF HISTAMINE ON THE ACID-BASE BALANCE.*

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(Received for publication, April 29, 1926.)

Dale and his collaborators (6, 14–18, 44) have shown that the more striking and clinically important effects following injection of histamine into cats and dogs resemble those of traumatic and surgical shock; *viz.*, oligemia due to loss of plasma from the circulation through the capillary walls, relaxation of the capillary bed, fall of blood pressure, etc. The parallelism appears sufficient to make an understanding of the various effects of histamine injection desirable as part of the general knowledge concerning the nature of shock.

We have accordingly studied one phase of the picture of histamine shock; *viz.*, the acid-base balance, a factor which has been found to be severely disturbed in traumatic shock and in some conditions which simulate it.

Fall of the carbon dioxide content of the blood and plasma appears to have been regularly observed in most of the types of shock that have been studied (6, 8–12, 21, 24–26, 30–33, 37, 38, 40). Such a fall can be caused either by hyperpnea, with blowing off of CO_2 from the blood, and a resultant H_2CO_3 deficit, or by acidosis caused by decomposition of blood bicarbonate by invading acids. In the former case the alkalinity of the blood, in terms of pH, is increased, in the latter it is usually diminished. Y. Henderson (30) at first considered the acid-base condition and chief causative factor in traumatic shock to be carbon dioxide deficit. Most other investigators have interpreted the condition as one of acido-

* Submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Faculty of Pure Science of Columbia University.

sis. In a few cases this interpretation has been confirmed by pH determinations in the blood plasma. Guthrie in traumatic shock caused in various ways (26), and Menten and Crile after manipulation of the intestines (39) observed a fall in plasma pH. Wittkower (53) and Zunz and La Barre (54) reported lowered plasma pH in anaphylactic shock in guinea pigs. De Waele reported increased plasma pH in shock caused by peptone injections in dogs (20).

It appears that in traumatic shock there is a condition of true acidosis, with lowering of both bicarbonate and pH in the blood plasma. As Dale (14) has pointed out, the acidosis is a consequence rather than a cause of the shock symptoms; they do not result from acidosis of the degree observed. However, dislocation of the acid-base balance, even though it does not play a dominant rôle in producing the symptoms, forms a frequent, if not a constant part of the disturbances involved, and must be considered in any attempt to draw the entire picture.

Reports of the effect of histamine shock on the acid-base balance have been conflicting. Wallace and Pellini (51) found that histamine injected subcutaneously caused an increase in plasma bicarbonate, but when injected intravenously under ether anesthesia caused a fall. Underhill and Ringer (45) concluded that the carbon dioxide content of the blood was not lowered as much by histamine as in other forms of shock. Hashimoto (27) found no consistent change in the plasma CO_2 content during histamine intoxication.

EXPERIMENTAL.

Dogs weighing between 11 and 16 kilos were used. They were fasted for 24 hours before the injection of histamine. The bladder was emptied by catheterization and a sample of blood was drawn just before the injection. Histamine was then injected subcutaneously, in varying amounts, and the acid-base balance was studied over a period of 6 hours thereafter, by means of blood and urine analyses. Blood was drawn by venepuncture at intervals, and the urine was collected by catheterization at the end of the 6 hour period.

Control experiments were performed, in which the acid-base balance was studied during a similar 6 hour period after a 24 hour

TABLE I.
Control Experiments. 6 Hour Periods following 24 Hour Fasts

TABLE I.

Control Experiments. 6 Hour Periods following 24 Hour Fasts.

Dog No.	Control No.	Body weight.	Time.	Blood.				Urine.			
				Plasma pH.	Blood CO_2 content.	Plasma CO_2 content.	Cell volume.	Volume.	pH	Titratable acid.	NH_3
		kg.			*vol. per cent*	*vol. per cent*	*per cent*	*cc.*		*cc. 0.1 N*	*cc. 0.1 N*
3	1	11.75	Beginning of 6 hrs.		29.8				5.15		
			End " 6 "		36.8			72	5.45*	78.5	104
3	2	11.75	Beginning of 6 hrs.	7.25	35.0				5.60		
			End " 6 "	7.25	37.5			36	5.60	51.2	56
6	1	11.5	Beginning of 6 hrs.						6.20		
			End " 6 "					67	5.55*	13.4	27
6	2	11.5	Beginning of 6 hrs.	7.42	41.9				5.60		
			End " 6 "	7.40	44.7			85	6.40		37
6	3	11.5	Beginning of 6 hrs.	7.36	40.9				5.30		
			End " 6 "	7.38	43.0			56	5.50	31.4	32
6	4	15.5	Beginning of 6 hrs.	7.25		57.4†	38.5		5.60		
			End " 6 "	7.33		63.2	34.0	81	6.50	16.2	41
6	5	15.7	Beginning of 6 hrs.	7.35		56.0	34.0		7.05		
			End " 6 "	7.31		55.8	34.3	61	6.20	32.3	34
6	6	15.7	Beginning of 6 hrs.	7.32		58.6	27.0		7.35		
			End " 6 "	7.31		59.8	28.0	97	6.40	15.6	33

* Bladder washed with boric acid (pH 5.2). All others washed with sterile 0.9 per cent saline.

† CO_2 capacity of plasma.

TABLE II.

Histamine Experiments. Study of 6 Hour Periods following Injection of Varying Amounts of Histamine.

Experiment No.	Dog No.	Injection No.	Dosage histamine base per kilo.	Body weight.	Time.	Blood.						Urine.			
						Plasma pH.	Blood CO_2 content.	Plasma CO_2 content.	Oxygen capacity.	Hemoglobin.	Cell volume.	Volume.	pH	Titratable acid.	NH_3
			mg.	*kg.*			*vol. per cent*	*vol. per cent*	*vol. per cent*	*per cent*	*per cent*	*cc.*		*cc. 0.1 N*	*cc. 0.1 N*
1	6	1	0.1	15.76	Before injection.	7.34		65.0*			31		6.95		
					5 min. after.	7.33		59.3			34				
					40 " "	7.29		64.1			34				
					6 hrs. "	7.27		60.9			31	89	7.40	16.6	33.6
2	6	2	1	15.7	Before injection.	7.37		58.4			34		6.25		
					38 min. after.	7.30		51.0			42				
					1½ hrs. "	7.28		46.9			45				
					6 " "	7.28		51.4			33	246	7.55	11.1	17.1
3	6	3	1	15.7	Before injection.	7.34		55.8			30				
					1½ hrs. after.	7.18		39.3			40				
					6 " "	7.23		56.6			33	135	7.65	5.4	6.4
4	6	4	1.15	11.5	Before injection.	7.27	39.0	(45.2)†	17.99	97.2	37.5		6.15		
					15 min. after.	7.19	34.5	(41.5)	26.40	142.7	36.0				
					45 " "		29.1	(33.6)	24.00	130.0					
					1½ hrs. "	7.26	36.0	(42.6)	22.85	123.5	32.5				
					3½ " "	7.30	42.7	(50.4)	19.69	106.4	37.5				
					6 " "	7.29	39.7	(45.7)	16.84	91.0	28.0	100	7.90	0.15	7.9

5	6	5	3	11.5	Before injection.	7.36	35.0	(41.0)	22.00	119.0	33.7		5.45		
					30 min. after.	7.31	24.5	(27.0)	23.68	128.0	38.0				
					1½ hrs. "	7.33	24.0	(26.5)	24.60	133.0	43.0				
					2½ " "	7.32	23.1	(25.3)	24.60	133.0	40.0				
					6 " "	7.35	36.2	(42.9)	23.11	125.0	39.0	37	8.00	1.7‡	4.9
					24 " "	7.42	37.9	(43.4)	16.84	91.0	23.5				
6	3	5	3	11.75	Before injection.	7.37	33.6	(37.0)	13.21	71.4	25.0		6.3		
					35 min. after.	7.21	30.0	(32.7)	14.00	75.7	27.0				
					1½ hrs. "	7.17	32.0	(35.5)	15.43	83.4	28.5				
					3 " "	7.38	29.2	(32.0)	15.43	83.4	26.0				
					6 " "	7.30	33.3	(36.3)	12.34	66.7		70	7.1		27.8

* CO_2 capacity of plasma.

† The figures in parentheses in this column were calculated from the blood CO_2 content according to the data of Peters, Bulger, and Eisenman (43).

‡ cc. 0.1 N HCl used in titration.

fast, when conditions were the same as on days when histamine was injected. In two of the control experiments the bladder was washed with boric acid (pH 5.2) after catheterization, as indicated in Table I. In all other experiments the bladder was washed with sterile 0.9 per cent saline solution. Precautions

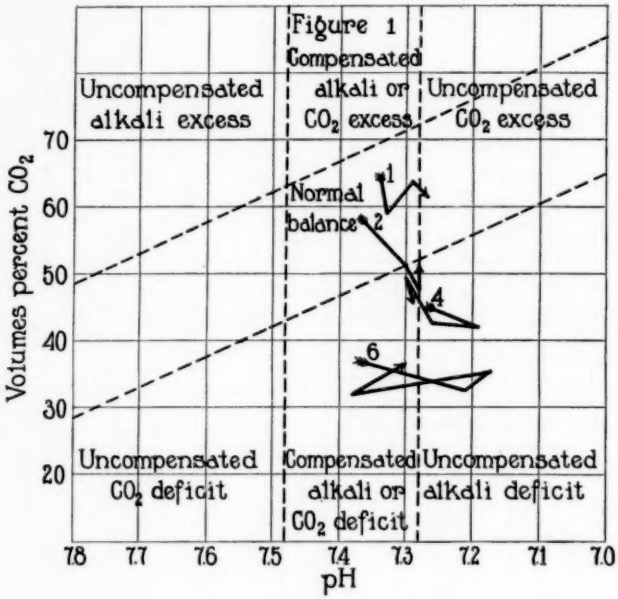

FIG. 1.

FIGS. 1 and 2. Variations in acid-base balance of plasma after histamine injection. The numbers on the curves indicate the number of the experiment in Table II, and are placed at the site of the preinjection estimations. The arrows indicate the direction taken during the course of the 6 hour period of each experiment.

were taken with regard to asepsis in the process, in order to prevent bladder infection and its effect on the reaction of the urine. Control days were interspersed between injection days as a further check on this factor.

No anesthetics were used, because anesthesia increases ap-

proximately ten-fold the shock produced by histamine on the organism (15) and also causes acidosis (3).

The acid-base balance was studied by determination of CO_2 and pH in the blood plasma, and of pH, titratable acid, and ammonia in the urine.

Preparation of Histamine Solution for Injection.—A Burroughs Wellcome and Company product, "ergamine," which is a prepara-

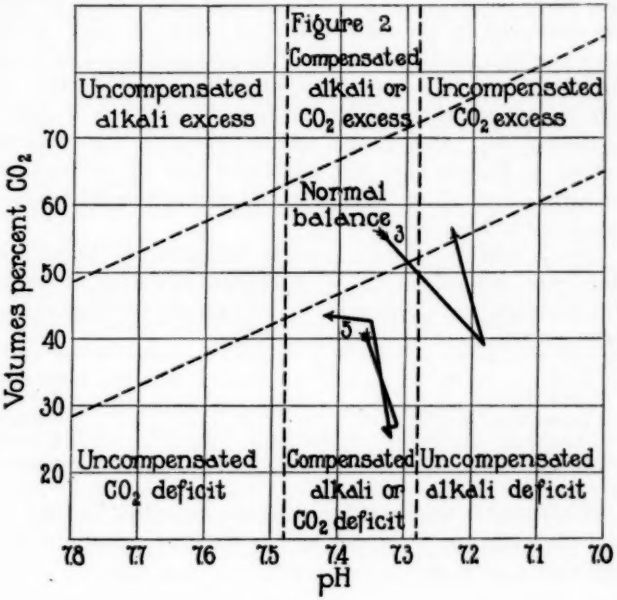

FIG. 2.

tion of histamine acid phosphate, was used. Analysis of this product indicated one-third of it to be histamine base. 0.4000 gm. of ergamine was dissolved in 0.9 per cent solution of sodium chloride, 0.128 gm. of sodium hydroxide was added to neutralize the phosphoric acid, and the solution was diluted to a volume of 20 cc. The pH of the solution was determined and was usually found to be 7.4. If it was not, the reaction was adjusted to 7.4. This solution was freshly prepared for each injection. 1 cc. contained

6.66 mg. of histamine base. All data are given in mg. of histamine base injected per kilo of body weight.

Methods.

Blood.—Blood samples were drawn from the jugular vein and collected under oil. Samples were taken as soon as possible for analyses. The pH of the blood plasma was estimated by the method of Cullen (13). The CO_2 content of the blood or plasma was estimated by the method of Van Slyke (49, 50). In those cases in which the CO_2 content of the plasma was not determined directly, it was calculated from the CO_2 content of whole blood and the oxygen capacity by the formula of Peters, Bulger, and Eisenman (43). Hemoglobin was estimated by the colorimetric method of Palmer (41) and sometimes also by the gasometric method of Van Slyke (46). The cell volume was determined by means of the hematocrit.

Urine.—The pH of the urine was estimated by the method of Henderson and Palmer (29), with changes in the indicators used.[1] The standard tubes were made up with ranges overlapping for the different indicators, as designated below.

	pH range.
Methyl red	4.4–6.0
Brom-cresol purple	5.2–6.8
Phenol red	6.8–8.4
Cresol "	7.2–8.8

The titratable acidity of the urine was estimated by the method of Folin (22), the ammonia by the aeration technique of Van Slyke and Cullen (48).

DISCUSSION.

The control experiments, Table I, showed in only one case variations in plasma pH exceeding the limit of experimental error. In this animal there was an increase of 0.08 pH during the 6 hour period.

Injection of histamine (up to 3 mg. per kilo), Table II, caused a variable fall in both pH and CO_2 content of the plasma, indicat-

[1] The recent method of Hastings, Sendroy, and Robson (28) had not yet been devised.

ing an uncompensated acidosis. The maximum drop in plasma CO_2 content was 16.5 volumes per cent in Experiment 3, Table II. The diminutions in pH and CO_2 content did not always run parallel. In Experiment 6, Table II, the CO_2 changes were slight, while the pH fell 0.20 but returned to the initial level more rapidly than some of the others. In Experiment 5 the CO_2 fell 11.9 volumes per cent in the blood while the pH fell only 0.05. In Experiment 3 the falls in CO_2 and pH were parallel, but the CO_2 had returned to normal in 6 hours while the pH had not yet returned to the initial level.

If the CO_2 is reduced primarily by overventilation the blood reaction swings to the alkaline side (19). Overventilation sufficient to cause an alkalosis from CO_2 deficit can be brought about in man by hot baths (5) and by subjection to lowered barometric pressure (34). The urine in these conditions has a high pH, and the acid excretion and ammonia output are low.

An examination of the urine after histamine injection showed high pH, in all the cases above 7.0, accompanied by a diminished ammonia output and a low excretion of titratable acid, compared with that of the normal control periods (Tables I and II).

From the findings in the blood alone, the condition appears to be one of acidosis. From the findings in the urine alone, the condition would readily be pronounced an alkalosis.

Charting the acid-base findings in the blood according to the method of Van Slyke (47) indicates that histamine shock shows a tendency towards an uncompensated alkali deficit, with final return to the preinjection condition (Figs. 1 and 2). The direction of the arrows in the charts indicates metabolic acid formation rather than a primarily respiratory change. The overproduction of non-volatile acid would cause normally an increased excretion of titratable acid and ammonia. The fact that the reverse occurs indicates a failure of the kidneys to respond to the threat of acidosis in a normal manner.

Experiments 2 and 3, Table II, show a definite tendency to diuresis, as compared with the controls. It is known that histamine injection increases the flow of the salivary, gastric, and pancreatic secretions, but it has not yet been shown that it increases excretion by the kidney. The diuresis may be due to the increased concentration of urea in the blood which has been

shown as a result of histamine injection in the accompanying paper (35).

A factor which would influence the acid-base equilibrium in histamine shock is the effect of histamine on gastric secretion. Ackman (1) showed, by injection of very small quantities of histamine (0.5 mg.) in human subjects, a marked increase in acid secretion in the stomach accompanied by an increase in the pH of the urine. Hashimoto (27), who gave larger injections of histamine to dogs, found an increased CO_2 capacity of the blood in only a few cases, despite the fact that his dogs all vomited as a result of the injection, and would therefore be expected to lose an even greater amount of HCl. He made no examination of the urine for acidity, but it is apparent that the kidney must have been excreting the excess alkali, or other metabolic changes were occurring to compensate the gastric effect. In the present report, dogs were used which showed a fairly good tolerance for histamine, and each was given a single small dose, so that in no case was there any vomiting, and the complications in acid-base balance of gastric origin were kept down as much as possible.

Another factor which might influence the acid-base balance in these experiments is exercise. Although the animals were kept as quiet as possible during the period of the experiment, there was in all cases a struggle just after the injection of histamine. A fall in CO_2 capacity and pH of the blood after short vigorous exercise has been reported by Arborelius and Liljestrand (2), and by Barr, Himwich, and Green (4), and a lowered CO_2 content has been found by Lundsgaard and Möller (36). Buell (7) found that when an animal struggled during an experiment the alkali reserve of the blood decreased. The urine during muscular exercise was found by Wilson, Long, Thompson, and Thurlow (52) to show an immediate increase in hydrogen ion concentration, acid, and ammonia excretion, with a return to normal values within 40 to 90 minutes after 1 or 2 minutes of exercise. The blood findings after histamine injection changed in the same direction, indicating acidification, as after exercise, but the urine examination showed the opposite results. If exercise had an effect on the acid-base balance in these experiments, it was overshadowed by other factors simultaneously at work.

There was in some of our experiments a considerable loss of

alkali in the urine. Gamble (23) has shown that the CO_2 tension and H_2CO_3 concentration of urine approximate those of the arterial blood plasma. Hence the bicarbonate content of the urine can be estimated by the Henderson-Hasselbalch equation, $pH = 6.1 + \log \frac{BHCO_3}{H_2CO_3}$. If the H_2CO_3 concentration is taken as 4.2 volumes per cent, an average normal value (23), the $BHCO_3$ would be 42 volumes per cent at pH 7.1, 84 volumes per cent at pH 7.4, 167 volumes per cent at pH 7.7, and 334 volumes per cent at pH 8.0. Doubtless the loss of bicarbonate through the urine was of some influence in creating the bicarbonate deficit in the plasma. However, the changes in the blood occurred so rapidly, reaching their maximum sometimes in a half hour, that excretion of alkali could hardly have been the main cause of the fall in plasma bicarbonate and pH. Even the bicarbonate output during the total period is not sufficient to account for much of the fall in plasma bicarbonate. In Experiment 4, Table II, the bicarbonate output was highest, because of the combined high urinary volume (100 cc.) and high pH (7.9). The bicarbonate content of such a urine would be about 265 volumes per cent, and its loss would occasion a fall of 11.6 volumes per cent CO_2 (that observed in plasma) in 2285 cc. of fluid. However, the fluid content of an animal of 11.5 kilos weight would be about 8 liters. Palmer and Van Slyke (42) have shown that bicarbonate changes in the body are distributed with some approximation to evenness throughout the body fluids. The observed bicarbonate output distributed over 8 liters, would cause a fall of only 3.3 volumes per cent CO_2 compared with the 11.6 volumes per cent fall observed.

SUMMARY.

Injection of histamine into dogs, in doses of 1 to 3 mg. per kilo, caused a fall of 5 to 16.5 volumes per cent in plasma CO_2, and a fall of 0.05 to 0.20 in plasma pH. The result is therefore a mild uncompensated acidosis, due to invasion of acid or loss of base in the blood.

The alkalinity of the urine excreted after histamine injection, reaching pH 7.1 to 8.0, was high compared with 5.4 to 6.9 before the injections. The kidneys failed to respond normally to the

internal change towards acidosis by increasing their acid output. The increased output of bicarbonate in the urine was a factor, but not the main one, in depressing the bicarbonate content of the blood.

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THE EFFECT OF HISTAMINE ON PROTEIN CATABOLISM.*

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Histamine shock has been shown by Dale and his collaborators (5–10, 26) to resemble other forms of shock, in consequence of which the study of histamine intoxication has acquired an interest in connection with the elucidation of the results of shock in general.

The experiments reported in this paper were performed partly to add to the data on the physiological effects of this type of shock, and partly to ascertain whether the effect of histamine intoxication on the endogenous protein catabolism resembled that of proteose intoxication. Whipple and Van Slyke (32) found that proteose intoxication, caused in a dog by closing an intestinal loop, or by injecting intravenously proteose prepared from the contents of a closed loop in another animal, is accompanied by an almost explosive destruction of body tissue, with sudden increase in the urea nitrogen content of the blood, and usually in the amino acid content. Haden and Orr (14) have also reported increases in non-protein and urea nitrogen in the blood of dogs after pyloric and intestinal obstruction. The observations of histamine in the intestine made by Abel and Kubota (1), Barger and Dale (4), Gerard (12), and Meakins and Harington (20), and the studies of the absorption of histamine in the intestine made by Mellanby (22) and Meakins and Harington (21) indicated the possibility that histamine absorption might play a part in such catabolic reactions.

* Submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Faculty of Pure Science of Columbia University.

EXPERIMENTAL.

Dogs weighing between 11 and 16 kilos were used. They were fasted for 24 hours before the injection of histamine. The bladder was emptied by catheterization and a sample of blood was drawn just before the injection. Histamine was then injected subcutaneously, in varying amounts, and the nitrogen metabolism was studied over a period of 6 hours thereafter by means of blood and urine analyses. Blood was drawn by venepuncture at intervals, and the urine was collected by catheterization at the end of the 6 hour period. In some cases the blood was examined also after a period of 24 hours.

Ether anesthesia was not used, because it increases approximately tenfold the shock produced by histamine on the organism (7), and also accelerates protein catabolism (25).

In only three experiments was any narcosis used. These are recorded in Fig. 13. Frequent and large samples of blood were drawn in these three experiments, and the femoral artery was cannulated under pantopon (a preparation of opium alkaloids) narcosis, with local anesthesia by novocaine. This form of narcosis appears to have little effect on the nitrogen catabolism.

Preparation of Histamine Solution for Injection.—A Burroughs Wellcome and Company product, "ergamine," which is a preparation of histamine acid phosphate, was used. Analysis of this product indicated one-third of it to be histamine base. 0.4000 gm. of ergamine was dissolved in 0.9 per cent solution of sodium chloride, 0.128 gm. of sodium hydroxide was added to neutralize the phosphoric acid, and the solution was diluted to a volume of 20 cc. The pH of the solution was determined and was usually found to be 7.4. If it was not, the reaction was adjusted to 7.4. This solution was freshly prepared for each injection. 1 cc. contained 6.66 mg. of histamine base. All data are given in mg. of histamine base injected per kilo of body weight.

Methods.

Blood Analyses.

Urea Nitrogen.—The urea nitrogen was determined by the method of Van Slyke and Cullen (31).

Free Amino Nitrogen.—10 cc. of blood were treated for 30 minutes with 1

cc. of a 10 per cent solution of Squibb's urease. The proteins were then precipitated by adding 5 per cent trichloroacetic acid to 100 cc. volume. 80 cc. of the filtrate were boiled 15 minutes over a free flame to decompose the trichloroacetic acid. The solution was then made alkaline to phenolphthalein by addition of a few drops of potassium carbonate solution, and was distilled *in vacuo* to a small volume. The residual solution was neutralized with acetic acid and diluted to a volume of 10 cc. The amino nitrogen was determined on 2 cc. portions by the nitrous acid method with the micro apparatus (28, 29).

Total Amino Nitrogen.—5 cc. of the solution prepared as above for amino nitrogen were mixed with 5 cc. of concentrated hydrochloric acid in a Pyrex test-tube. The tube was covered with an inverted short, wider tube and digested in a boiling water bath for 24 hours to set free amino groups involved in peptide linkings. The solution was then evaporated almost to dryness on the water bath in a small evaporating dish, neutralized with sodium hydroxide, and diluted to a volume of 5 cc. Amino nitrogen determinations were made as above, giving the preformed free amino nitrogen plus the peptide nitrogen freed by the acid hydrolysis.

Peptide nitrogen was calculated by subtracting the "free amino nitrogen" from the "total amino nitrogen" obtained after acid hydrolysis.

Hemoglobin.—Some of the hemoglobin determinations were made according to the gasometric method of Van Slyke (30) and some by the colorimetric method of Palmer (24), which was checked against the gasometric method.

The cell volume was determined by means of the hematocrit.

Urine Analyses.

Urea Nitrogen.—Studies of urea excretion were made over a period of 6 hours after the injection of histamine. The urea nitrogen of the urine was determined by the method of Van Slyke and Cullen (31).

Urea Formed in the Body.—The total amount of urea formed in the body during a period of 6 hours after the injection of histamine was calculated by assuming that the body contains 700 cc. of fluid per kilo of body weight, and that urea is uniformly distributed throughout these fluids, as was found by Marshall (19) to be approximately the case. The total amount of urea nitrogen formed during the period of the experiment was calculated as

$$\underbrace{0.7\,W(B_2 - B_1)}_{\text{Increase in total urea content of body.}} + \underbrace{\text{urine urea}}_{\text{Urea excreted during experiment.}} = \text{urea formed during experiment.}$$

W = body weight in kilos.
B_1 = blood urea per liter at start of experiment.
B_2 = " " " " " end " "

The animals in which the total urea formation was studied were controlled by studies of the urea formation rate during a similar 6 hour period, after a 24 hour fast, when conditions were the same as on days when histamine was injected. The results are given in Tables I to IV.

TABLE I.

Dog 6. Control Experiments. Urea Formation during 6 Hour Period after 24 Hour Fast.

Time.	Control No.	Body weight.	Blood urea N per 100 cc.	Urine.		Urea N formed in body.
				Volume.	Urea N.	
		kg.	*mg.*	*cc.*	*mg.*	*mg.*
Beginning	1	11.5	8.87			
End			8.78	67	571	565
Beginning	2	11.5	11.03			
End			10.55	85	760	721
Beginning	3	11.5	8.49			
End			9.41	56	427	501
Beginning	4	15.5	7.28			
End			7.00	81	438	405
Beginning	5	15.7	8.87			
End			8.03	61	613	521
Beginning	6	15.7	9.91			
End			7.95	97	585	370
Average					566	514

DISCUSSION.

Blood Urea.—All curves show an increase in urea nitrogen in the blood during a 6 hour period after injection of histamine. This increase varies with the amount of histamine injected. After injection of 0.1 mg. per kilo of body weight (Fig. 1) the increase was 8 per cent of the blood urea nitrogen concentration observed before the injection. After injection of 1 mg. per kilo (Figs. 2, 3, and 4) the increase was 25 to 79 per cent. After injection of 3 mg. per kilo (Figs. 5, 6, and 7) the increase was about 100 per

cent. After injections of 5.6 to 8.6 mg. per kilo (Figs. 8, 9, and 10) there was an increase in 6 hours of 105 to 150 per cent. Except

TABLE II.

Dog 6. Urea Formation during 6 Hour Period after Injection of Varying Amounts of Histamine.

Time.	Injection No.	Dosage histamine base per kilo.	Body weight.	Blood urea N per 100 cc.	Urine.		Urea N formed in body.
					Volume.	Urea N.	
		mg.	*kg.*	*mg.*	*cc.*	*mg.*	*mg.*
Beginning	1	0.1	15.76	11.95			
End				10.91	89	953	839
Beginning	2	1	15.7	12.96			
End				16.32	246	821	1193
Beginning	3	1	15.7	8.00			
End				14.28	135	471	1161
Beginning	4	1.15	11.5	9.7			
End				13.9	100	523	861
Beginning	5	3	11.5	8.30			
End				16.55	37	270	936
Average						608	998

TABLE III.

Dog 3. Control Experiments. Urea Formation during 6 Hour Period after 24 Hour Fast.

Time.	Control No.	Body weight.	Blood urea N in 100 cc.	Urine.		Urea N formed in body.
				Volume.	Urea N.	
		kg.	*mg.*	*cc.*	*mg.*	*mg.*
Beginning	1	11.75	14.35			
End			9.61	72	1034	691
Beginning	2	11.75	10.30			
End			8.44	36	531	378

after the smallest injections, the blood urea was still rising at the end of the 6 hour period. The maximum increases therefore

probably were greater than those recorded. Analysis of the blood 24 hours after injection always showed a return to normal.

The results of Whipple and Van Slyke (32) after the injection of proteose prepared from the material obtained from the closed loop of the small intestine of a dog showed an increase of 112 per cent in the urea nitrogen of the blood 5 hours after an injection of 108 mg. of the preparation per kilo of body weight. These results obtained after the injection of proteose correspond to the results obtained after the injection of the larger doses of histamine (5.6 to 8.6 mg. per kilo), as shown in Fig. 14.

Rate of Urea Formation.—The nitrogen catabolism from the standpoint of urea formation was further studied in order to assure ourselves that the increase of urea in the blood was not merely an accumulation due to a retention on the part of the kidney.

TABLE IV.

Dog 3. Urea Formation during 6 Hour Period after Injection of Histamine.

Time.	Injection No.	Dosage histamine base per kilo.	Body weight.	Blood urea N per 100 cc.	Urine.		Urea N formed in body.
					Volume.	Urea N.	
		mg.	*kg.*	*mg.*	*cc.*	*mg.*	*mg.*
Beginning.....	5	3	11.75	10.54			
End.....				19.20	70	627	1331

By the formula given above, the urea formed in the 6 hour period was calculated as the sum of the amount excreted plus the increase in the urea content of the body, both after injection of histamine and in the normal control periods on the same animals. Tables I, II, III, and IV show that the rate of urea formation was almost doubled by injection of 1 to 3 mg. of histamine per kilo of body weight.

The rate of urea excretion did not rise in proportion to the increased production of urea in the body, as it would under normal conditions (19, 3). The acceleration of urea formation increased only the blood urea concentration, instead of dividing its effect between blood urea content and urea output rate, as would normally be the case. The renal function therefore appears to have been somewhat depressed by the histamine intoxication.

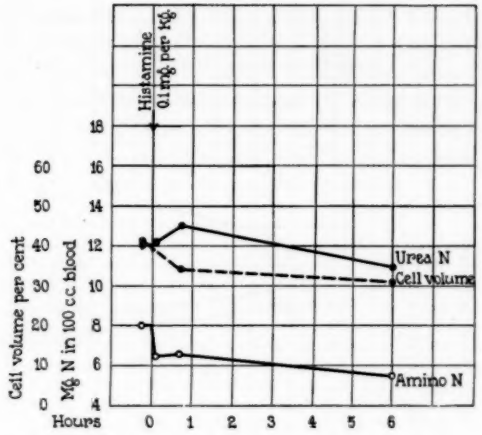


FIG. 1. Dog 6, Injection 1, weight 15.76 kilos.

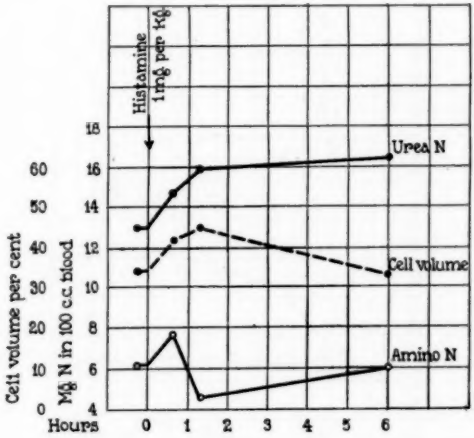


FIG. 2. Dog 6, Injection 2, weight 15.7 kilos.

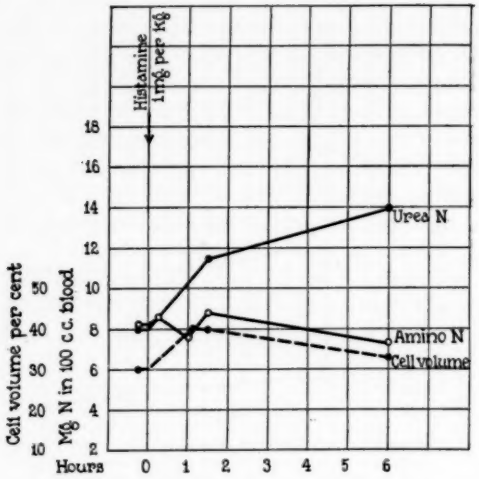


FIG. 3. Dog 6, Injection 3, weight 15.7 kilos.

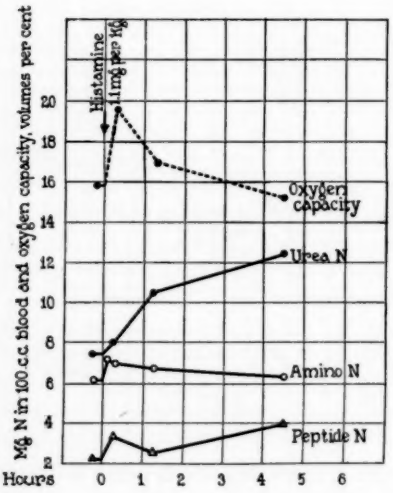

Histamine 1 mg. per Kg.
Oxygen capacity
Urea N
Amino N
Peptide N
Mg. N in 100 c.c. blood and oxygen capacity, volumes per cent
Hours
0 1 2 3 4 5 6

FIG. 4. Dog 3, Injection 3, weight 11.75 kilos.

Blood Amino and Peptide Nitrogen.—The changes in the amino and peptide nitrogen of the blood, with a few exceptions, were slight. An increase in both usually occurred within the first 2 hours after injection, with a return to normal at the end of the 6 hour period. Since the amino nitrogen is more concentrated in the cells than in the plasma (13), the question arose as to whether the increase of amino nitrogen in the early period in a few cases

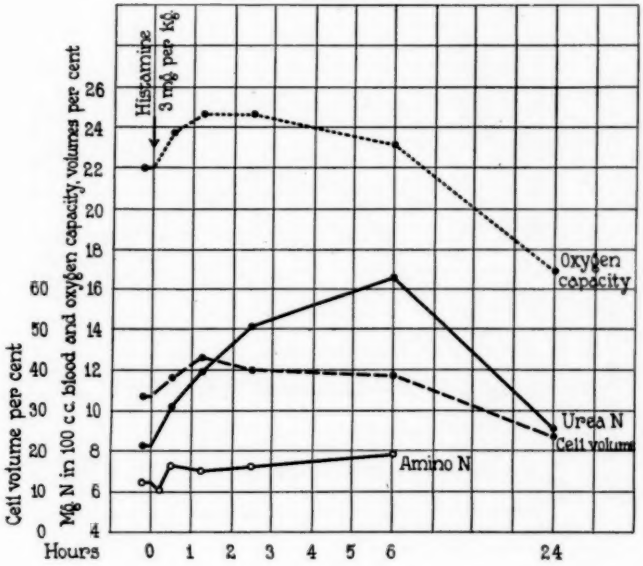

Histamine 3 mg. per kg.
Cell volume per cent
Mg. N in 100 c.c. blood and oxygen capacity, volumes per cent
Oxygen capacity
Urea N
Cell volume
Amino N
Hours
0 1 2 3 4 5 6 24

FIG. 5. Dog 6, Injection 5, weight 11.5 kilos.

after fairly large injections of histamine might not be due to the concentration of the blood as indicated by the increase in cell volume.

Accordingly three dogs (Fig. 13) were given large doses of histamine (3.7 to 7.5 mg. per kilo) after injection of pantopon and cannulation of the femoral artery under local anesthesia, in order that large and frequent samples of blood might be obtained during the early period after injection, and that the amino nitrogen

might be determined both in whole blood and in plasma. The curves show no relationship between cell volume and amino nitrogen changes. The latter appear to represent genuine changes in the amount of amino acids in the circulation.

The increase of 2.7 mg. of amino nitrogen and 3 mg. of peptide nitrogen per 100 cc. of blood found by Whipple and Van Slyke (32) 5 hours after injection of 108 mg. of proteose per kilo cor-

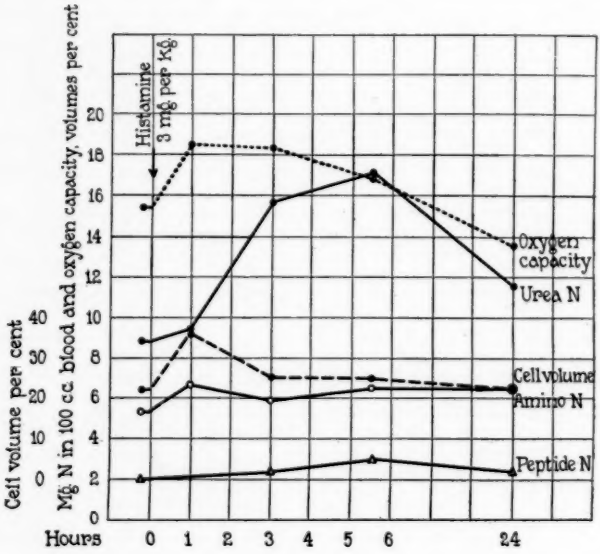

Cell volume per cent
Mg. N in 100 cc. blood and oxygen capacity, volumes per cent
Histamine 3 mg. per Kg.
Oxygen capacity
Urea N
Cell volume
Amino N
Peptide N
Hours
0 1 2 3 4 5 6 24

FIG. 6. Dog 3, Injection 4, weight 11.75 kilos.

responds to the average increase found after injection of large amounts of histamine, except that the rise in amino nitrogen appears to occur and subside more quickly in histamine shock.

The findings for urea and amino nitrogen are similar to those reported by Hashimoto (16) after these experiments were completed.

Toxic Symptoms.—The toxic symptoms after injection of histamine appear to be somewhat different from those reported by

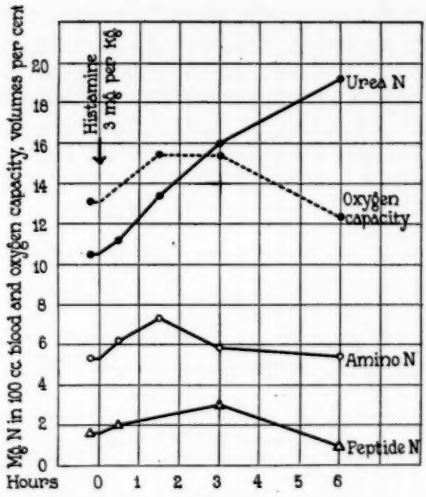

Mg. N in 100 cc. blood and oxygen capacity, volumes per cent
Histamine 3 mg. per Kg.
Urea N
Oxygen capacity
Amino N
Peptide N
Hours
0 1 2 3 4 5 6
0 2 4 6 8 10 12 14 16 18 20

FIG. 7. Dog 3, Injection 5, weight 11.75 kilos.

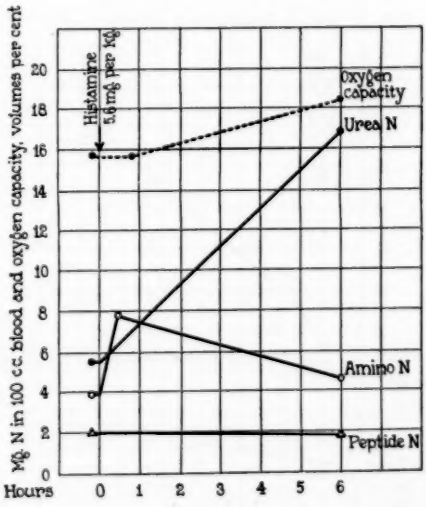

Mg. N in 100 cc. blood and oxygen capacity, volumes per cent
Histamine 5.6 mg. per Kg.
Oxygen capacity
Urea N
Amino N
Peptide N
Hours
0 1 2 3 4 5 6
0 2 4 6 8 10 12 14 16 18 20

FIG. 8. Dog 3, Injection 2, weight 11.75 kilos.

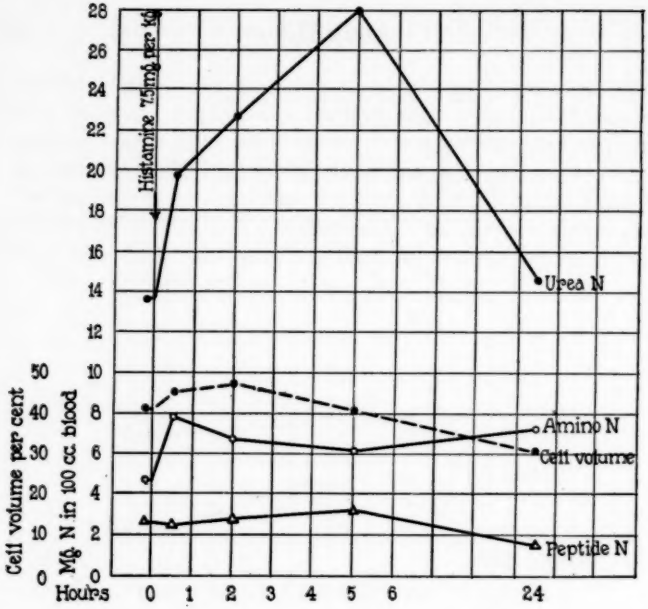


FIG. 9. Dog 3, Injection 1, weight 11.75 kilos.

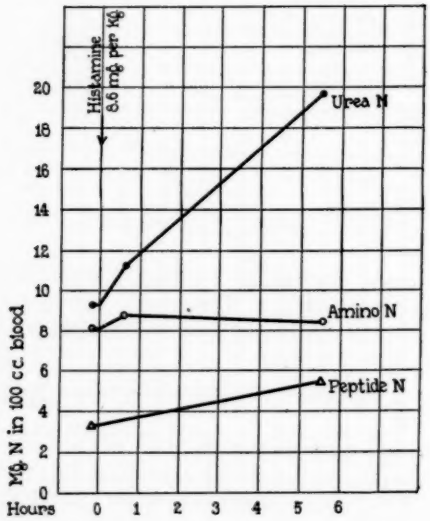


FIG. 10. Dog 4, weight 16.25 kilos.

Whipple and Van Slyke (32) after proteose injection under ether anesthesia. Their animals developed diarrhea, vomiting, and collapse, and lived for some hours. Most of our dogs showed signs of drowsiness for 3 or 4 hours, and then rapidly returned to normal. Only one (Dog 4, Fig. 10) vomited. Two (Dogs 1 and 9, Figs. 11 and 12) went into complete collapse, with severe

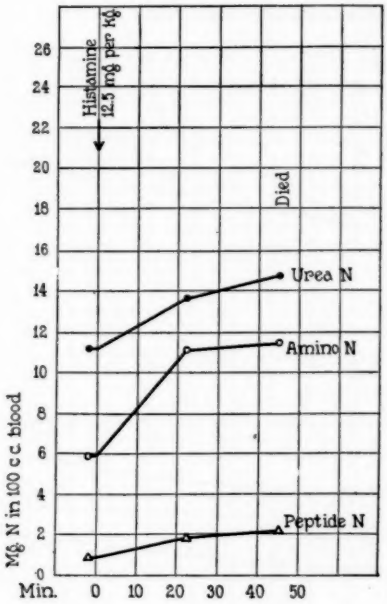


FIG. 11. Dog 1, weight 13.25 kilos.

diarrhea, immediately after their injections, and died in 45 and 110 minutes respectively.

Non-Specific Nature of Acceleration of Protein Catabolism.—Acceleration of protein catabolism obtained after injection of histamine in dogs is similar to that obtained by Whipple and Van Slyke (32), after injection of proteose, and by other authors in cases of anaphylactic (17, 18, 27, 33) and traumatic (2, 11, 23)

shock, and commonly as the result of intoxication in infectious diseases. The rise in blood urea after the proteose and histamine injections is not essentially different from that obtained after a heavy feeding of meat (32); the amount of urea formed does not reach a toxic height. The amino and peptide nitrogen changes in these experiments correspond with the slight rise in the amino nitrogen reported by other authors (17, 18, 32, 33) during anaphylactic and proteose shock.

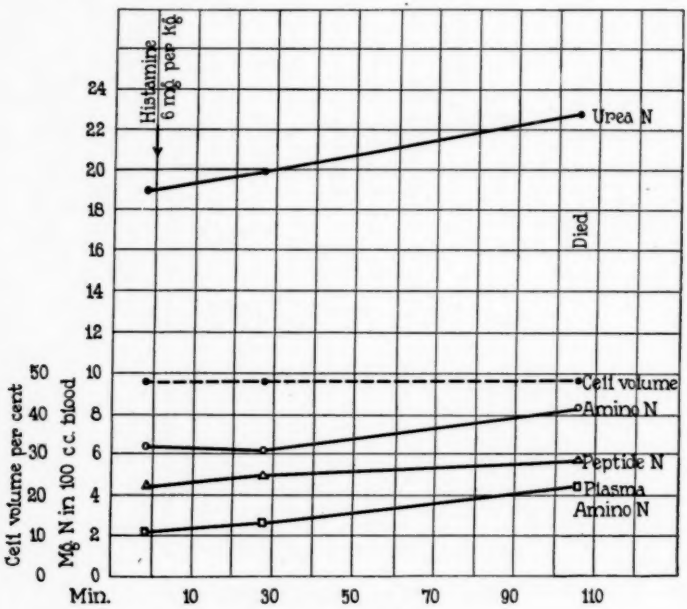


FIG. 12. Dog 9, weight 18.8 kilos.

The similarity in the results obtained after histamine injection and after proteose injection suggests the possibility of the presence of histamine in the proteose material. However, as an increased protein catabolism is a general feature of so many forms of shock as well as of such unrelated toxic conditions as those accompanying various infectious diseases and burns, the results seem far from specific for the presence of histamine. The results of Hanke

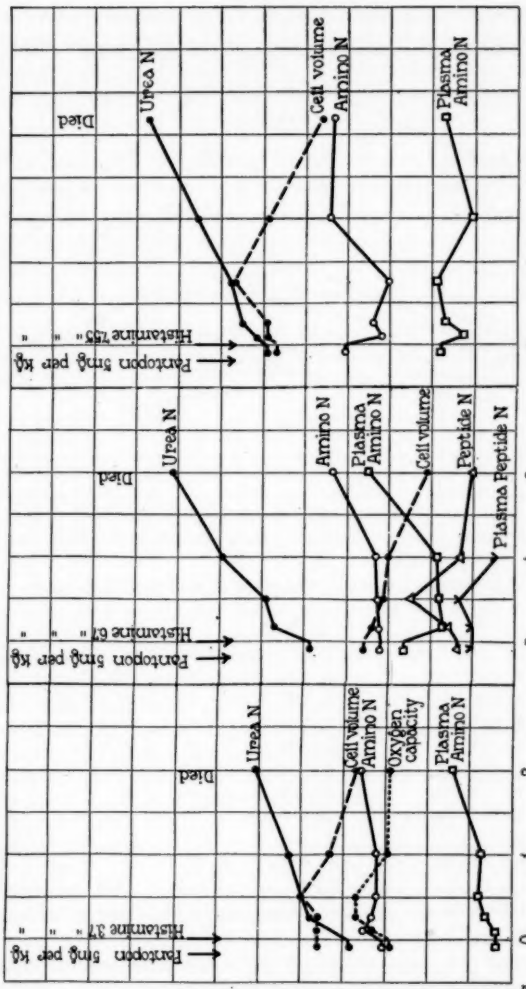
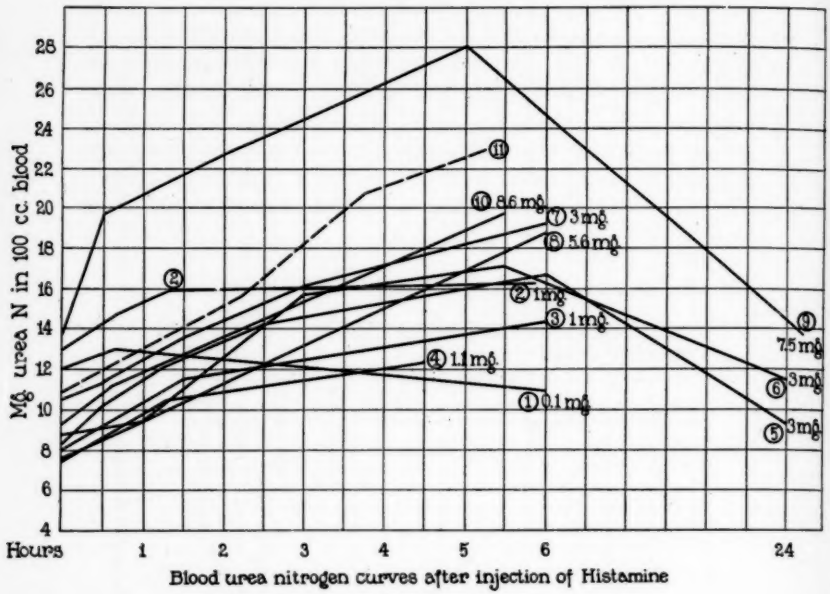


FIG. 13. (*a*) Dog 5, weight 25 kilos. (*b*) Dog 3, Injection 6, weight 11.75 kilos. (*c*) Dog 7, weight 10.75 kilos.



Blood urea nitrogen curves after injection of Histamine

FIG. 14.

Curve 1. Dog 6, Injection 1; 0.1 mg. histamine per kilo.
" 2. " 6, " 2; 1.0 " " " "
" 3. " 6, " 3; 1.0 " " " "
" 4. " 3, " 3; 1.1 " " " "
" 5. " 6, " 5; 3.0 " " " "
" 6. " 3, " 4; 3.0 " " " "
" 7. " 3, " 5; 3.0 " " " "
" 8. " 3, " 2; 5.6 " " " "
" 9. " 3, " 1; 7.5 " " " "
" 10. " 4, 8.6 " " " "
" 11. Proteose curve, 260 cc. of solution = 1300 mg. substance. Weight 12 kilos = 108 mg. per kilo. Whipple and Van Slyke (32), Dog 18–12.

and Koessler (15), who obtained the same shock effects with histamine-free peptone as with Witte's peptone, appear to be stronger evidence against histamine as a factor than our results are for it. If we had found histamine shock to be unaccompanied by increased protein catabolism, the fact would have excluded

histamine as a possible cause of proteose shock. We have failed to exclude it, but we do not consider that our data constitute evidence for it.

SUMMARY.

Histamine injections of 1 to 8 mg. per kilo produce in fasting dogs increase in endogenous protein catabolism, lasting for periods of several hours and proportional in intensity to the dose administered. The effect on protein catabolism is similar to that caused by injection of Whipple's proteose from intestinal loops, and to that caused by various other forms of acute intoxication.

Histamine injection affords an easily controlled means of producing an acute transient condition of tissue destruction in the body.

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